Development of a Multilayer Emulsion System for the Protection of Polyunsaturated Fatty Acids in Flaxseed Oil

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by

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(Roll Number: 614FP1001)

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under the supervision of

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This is to certify that the work presented in the dissertation entitled *Development of a Multilayer Emulsion System for the Protection of Polyunsaturated Fatty Acids in Flaxseed Oil* submitted by *S. Sivapratha,* Roll Number 614FP1001, is a record of original research carried out by her under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology (Research)* in *Food Process Engineering.* Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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To my parents...

Declaration of Originality

I, *S.Sivapratha*, Roll Number *614FP1001* hereby declare that this dissertation entitled *Development of a Multilayer Emulsion System for the Protection of Polyunsaturated Fatty Acids in Flaxseed Oil* presents my original work carried out as a masters student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the section "References". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

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Abstract

Among the various components of food, fats are considered as an offender for cardiovascular problems in human beings. However, many polyunsaturated fatty acids are identified as being beneficial to health. Fats are an integral component of many food system and is difficult to eliminate its presence completely. There is an increased interest in substituting the harmful fats with health promoting polyunsaturated fatty acids including ω-3 fatty acids. All PUFAs are characterized with unsaturated chemical bonds that render them unstable due to oxidative degradation. The susceptibility of ω-3 fatty acids to lipid oxidation has hindered the incorporation of these heart healthy polyunsaturated fatty acids into healthful foods and beverages. The contributing factors to oxidation include metal pro-oxidants, heat and light. Therefore, emulsions are devised as a means of protecting the ω-3 fatty acids in oil.

An attempt is made to encapsulate the advantageous flaxseed oil with the aid of multilayer oil-in-water emulsion. This is achieved in a conventional primary emulsion when oil/water interface morphology is modified using a multitude of surface active biopolymers. It is desired that the biopolymers interact constructively and form an emulsion that is resistant to flocculation and aggregation with increasing storage time, salt concentration and temperature. The aim of this research is to create a novel tertiary and secondary emulsion and test its suitability for protecting the oil phase from stress factors (temperature and salt variation) and chemical (oxidative) breakdown.

In this study, plant based flaxseed oil rich in ω-3 fatty acids were dispersed into primary, secondary and tertiary emulsion system. A primary emulsion (1% flaxseed oil, 0.4 % sodium caseinate) containing sodium caseinate stabilized cationic droplets were prepared by homogenizing flaxseed oil as oil phase and sodium caseinate solution as aqueous phase in an ultrasonicator. A secondary emulsion (1% flaxseed oil, 0.4% sodium caseinate + 0.25% sodium alginate) comprising of sodium caseinate- sodium alginate anionic droplets were produced by diluting appropriate primary emulsion with alginate solution. Further, tertiary emulsion (1% flaxseed oil, 0.4% sodium caseinate + 0.25%

sodium alginate + 0.25 % chitosan) composed of sodium caseinate- sodium alginate-chitosan coated cationic droplets were produced by diluting suitable secondary emulsion with chitosan solution. The resistance of primary, secondary and tertiary emulsions with the same lipid concentration to destabilization by thermal treatment (30−90 °C for 30 min), high sodium chloride concentration (≤ 70 mM NaCl) and oxidative degradation (hydroperoxide concentration and TBARS) were determined. The results showed that secondary emulsions could resist variation in environmental stresses of salt and heat as well as protect the oil phase from decomposition better than primary and tertiary emulsions. Interfacial engineering could be used to design emulsion system with desirable characteristics.

Keywords: ***Tertiary emulsion; α-linolenic acid; hydroperoxide; turbidity; secondary emulsion; TBARS***

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Chapter 1

Introduction

In the modern world with lack of time for food preparation, processed foods are largely replacing the traditional ones. Several manufactured foods are consumed as an alternative to meet the energy and nutritional requirements. Such factory-made foods are characteristically composite, multi-constituent colloid systems. Their properties are dictated by the molecular characteristics of the ingredients used and interaction among the numerous molecules. It is desired that these products have appreciable stability, texture and nutrition. Therefore, a thorough understanding about the colloidal phenomenon including aggregation and adsorption is utterly necessary. Regarding emulsion, which is a type of colloidal dispersion, study of structure, composition and interaction is essential to offer stability to the food system (Berendsen et al., 2014; Dalgleish, 1997).

Emulsions essentially comprise of two phases- a water phase and an oil phase. The boundary of oil and water phases is called as an interface. Several types of emulsions are known to exist including conventional oil-in-water and water-in-oil emulsions, multiple emulsions, multilayer emulsions, Pickering emulsions, filled hydrogels and solid lipid particles (McClements, 2012). Stable emulsions are those that resist transformation in physicochemical properties with time. Emulsions are thermodynamically unstable systems having the innate nature to separate into oil and water phases (McClements, 2007). Amphiphilic molecules called emulsifiers are added to bring down the surface tension between water and oil phases (Berendsen et al., 2014; Dickinson, 1993). With the use of emulsifiers, kinetically stable emulsions can be prepared (McClements, 2004; Rousseau, 2000). Coalescence, phase inversion, creaming, flocculation, sedimentation and Ostwald ripening are instability phenomena that occur over time and require prevention (McClements, 2007; Tadros, 2004). The emphasis on understanding emulsion structure, formation and stability is made as they can be designed as a vehicle for carrying minerals, microorganisms, carotenoids, oils, phenolic compounds, amino acids and vitamins. Carrier systems such as emulsions are formed for incorporation into daily foods like beverages, sauces and dips (Jiménez-Colmenero, 2013).

Flaxseed (*Linum usitatissimum*) oil is rich in polyunsaturated fatty acids (PUFA) specifically ω-3 fatty acids. The notable ω-3 fats occurring in food include

eicosapentaenoic acid, docosahexaenoic acid and α- linolenic acid. Majority of these ω-3 fats are found in fish fat and plant sources are lean. Flaxseeds contain 58.5 ± 0.1 % (w/w) α-linolenic acid out of the total crude oil expelled out of seeds, which implies that flaxseed oil is a good source of ω-3 fatty acids (Goyal et al., 2014; Shim et al., 2015). There are indefinite health benefits reaped from the inclusion of ω-3 fats in human nutrition. Some of them are decrease in the likelihood of mental sickness, improvement of heart health, enhanced brain and eye performance, defense against hypercholesterolemia, mood disorders, retinal diseases and inflammatory bowel diseases (Goyal et al., 2015; Julio et al., 2015; Rabetafika et al., 2011; Riediger et al., 2008; Siddiqui et al., 2004; Taherian et al., 2011). Consequently, the incorporation of ω-3 fatty acids in daily food is highly desirable.

The inclusion of ω-3 fatty acids in food suffers a major hindrance which is oxidation. Since, the ω-3 fats are PUFA with multiple double bonds, they are highly prone to oxidative degradation. The degraded materials give out off-flavours thus hampering the organoleptic quality of food and nutritional benefits are also depleted (Dlugogorski et al., 2012; Giroux et al., 2010). In addition, adverse genotoxic and cytotoxic effects could be induced in humans due to the formation of degradation products like malonaldehyde, acrolein, hexanal and propanal (Fang et al., 1996; Giroux et al., 2008; Taherian et al., 2011). The use of emulsions with complex interfaces is suggested as a means of protecting and delivering vulnerable bioactive compounds like ω-3 fatty acids (Liu et al., 2016c; McClements, 2010).

Multilayer emulsions are systems with complex interface as it has more than one layer constituting the interface. The emulsion formed using one biopolymer as an emulsifier is referred to as a primary emulsion. When two biopolymers, one on top of another, interact electrostatically at the emulsion interface, secondary emulsion is known to have formed. Addition of a third biopolymer layer on the secondary emulsion yields tertiary emulsion (Aoki et al., 2005; Guzey and McClements, 2006; Tokle et al., 2013). Electrostatic layer-by-layer (lbl) deposition approach is usually employed to form multi-layer emulsions (Gudipati et al., 2010).

Interface of a multilayer emulsion can be formed using a wide array of proteins, polysaccharides, phospholipids and surfactant combinations (Gudipati et al., 2010; Güzey and McClements, 2006; Mao et al., 2013; Ogawa et al., 2004). Proteins and

polysaccharides are good emulsifiers by themselves (Najafi et al., 2016). However, a single protein or polysaccharide emulsifier is rarely capable of having all the desired attributes of a food emulsion. Therefore, the beneficial traits of several biopolymers can be combined by creating multilayer interfaces (Aoki et al., 2005; Fioramonti et al., 2015; Güzey and McClements, 2006; Liu et al., 2015b; Ogawa et al., 2004). In this work, a protein, sodium caseinate and two polysaccharides, chitosan and sodium alginate were employed to form tertiary emulsion. Trilayer emulsions were prepared from sodium caseinate coated oil droplets, above which secondary layer sodium alginate and tertiary chitosan layers were sequentially adsorbed by lbl deposition method. In addition, secondary emulsion stabilized with bilayer membrane consisting of internal sodium caseinate and external sodium alginate and primary emulsion coated by sodium caseinate were compared with tertiary emulsion.

There are some research work on formation and stability assessment of tertiary (Aoki et al., 2005; Gu et al., 2005; Noshad et al., 2016; Ogawa et al., 2004; Xu et al., 2016a) and secondary emulsions (Ogawa et al., 2003; Perrechil and Cunha, 2013; Qiu et al., 2015a; Wei and Gao, 2016). However, little has been explored about how well these composite interface can protect the lipid phase for oxidative degradation (Gudipati et al., 2010) . This work has the following objectives:-

(1) Formation of a novel flaxseed oil-in-water tertiary emulsions using sodium caseinate, sodium alginate and chitosan as interface material.

(2) Physical characterization of prepared tertiary emulsion with respect to secondary and primary emulsion

(3) Effect of emulsion stress factors like varying temperature and sodium chloride (NaCl) concentration on size of emulsions

(4) Comparative study of oxidative stability of primary, secondary and tertiary emulsions

At the end of this work, the principles and complexities underlying the development and stability of primary, secondary and tertiary oil-in-water emulsion was understood. The solubility and behavior of biopolymers at various pH, influence of buffer on emulsions, formation of standard curve and estimation of TBARS and hydroperoxide concentration in a dispersed system was known.

Chapter 2

Literature review

2.1. Emulsification

Formation of emulsion with desired property depends upon a variety of interrelated factors including viscosity and composition of continuous and dispersed phases, the choice of surface-active material and emulsifying technique. Emulsification is achieved mostly by using energy intensive mechanical shearing operations such as colloid milling, high-speed mixing, microfluidization, high pressure homogenization and ultrasonication. The choice of emulsifying technique dictates the droplet size and its distribution (Berendsen et al., 2014; Purwanti et al., 2016). Ultrasonication is a convenient method to produce emulsions with small droplet size and lower polydispersity in view of the fact that it consumes less energy and surfactant (Hashtjin and Abbasi, 2015). On the contrary, the technique suffers certain disadvantages such as probable titanium contamination from probe tips, complication in scaling up and acceleration of chemical degradative reactions (Quintanar-Guerrero et al., 1998).

Conventionally, emulsions are formed by homogenizing a water phase, an oil phase and emulsifiers. During homogenization, disruption of emulsion droplets is assisted by emulsifier that reduces oil/water tension at the interface. In addition, emulsifier forms a protective membrane at the oil/water interface (Aoki et al., 2005). For a long time, a range of surface active compounds are used for stabilizing the oil/water interface including proteins (Horn et al., 2013; Ji et al., 2015), polysaccharides (Sukhotu et al., 2016; Zhang et al., 2016) and phospholipids (Hu et al., 2016; Züge et al., 2013). These biopolymers must assist formation of small spherical droplets of oil-in-water, align themselves at the oil/water interface and reduce the surface tension between the two phases. Also, the biopolymer should impart charge, thus preventing droplet aggregation by mutual repulsion.

2.2. Conventional emulsifiers and their limitations

Food hydrocolloids have the ability to behave as emulsifying agents. Hydrocolloids are hydrophilic long chain polymers that form viscous dispersions in water. Several starches, gums and proteins are hydrocolloids (Saha and Bhattacharya, 2010). Their

emulsifying properties depend upon molecular characteristics such as charge, hydrophobicity, molar mass, concentration, conformation and branching and bulk physicochemical characteristics including gelling, thickening and light scattering. Food hydrocolloids in emulsion system prevent the creaming of oil droplets by gelling the aqueous phase. Also, many hydrocolloids are dietary fibers that adds to human nutrition (Chung et al., 2013)

Proteins extracted from various natural sources have the ability to form stable emulsions with desirable attributes (Taherian et al., 2011). Proteins have a hydrophobic region and a hydrophilic region in the same molecule, thus assisting their spontaneous alignment on the oil/water interface. Proteins being amphiphilic molecules can reduce interfacial tension by adsorbing to the surface. In addition, they also form film bringing about repulsion between droplets by electrostatic and steric effects (Calero et al., 2013; Cerimedo et al., 2010). In the presence of gums, the oil phase of the oil-in-water emulsion has restricted mobility as the hydrocolloid absorbs water and increases the viscosity of the continuous medium (Najafi et al., 2016). Phospholipids from natural origin and partially modified forms are amphiphilic molecules capable of being used as emulsifiers and foaming agents (Letyagina et al., 2014).

The most widely used class of biopolymer as emulsifier in the food sector are the proteins (Cerimedo et al., 2010). In general, proteins themselves are good surfactants and can act as good emulsifiers (Najafi et al., 2016). For instance, principal milk protein, caseins form a thick fluid interface with the casein molecule protruding about 13–15 nm out into the bulk medium. The structure of casein molecules forming interfacial membrane have several loops and tails extended into the aqueous phase conferring stability against coalescence and aggregation. Casein based emulsions are quite stable unless the steric hindrance is collapsed due to solvent precipitation of protein with ethanol, inclusion of calcium ions, proteolysis or pH reduction. On the other hand, a thin layer of 2-3 nm is formed by whey protein (Dickinson, 2008; Wooster and Augustin, 2006) and principal stabilization of emulsion occurs by electrostatic interaction in emulsions formed by whey protein isolate (Tcholakova et al., 2005). With meagre amount of protein protruding into the bulk, whey protein forms a thin dense interface which could be disintegrated by the presence of electrolyte or when pH is close to the protein's isoelectric pH (Wooster and Augustin, 2006). Therefore, the surface activity of proteins are vulnerable to changes in

pH, salt and ionic strength (Kasran et al., 2013; Qiu et al., 2015c; Yadav et al., 2010; Zhang et al., 2012).

Furthermore, polysaccharides used as emulsifiers confer stability to emulsions over a broad range of pH and ionic strength (Dickinson, 2008). Protein coated emulsions are stabilized electrostatically, while polysaccharide coated emulsions are steric stabilized (Salminen and Weiss, 2014). The surface action of carbohydrates originates from its molecular composition and structure. In certain biopolymers like modified starch or cellulose, hydrophobic groups are chemically attached to the hydrophilic polysaccharides, making the resulting molecule amphiphilic. On the other hand, polysaccharide emulsifiers like gum Arabic, corn fiber gum and sugar beet pectin have a minor protein fraction that confers hydrophobic nature. In this way, their structural composition makes the molecule amphiphilic. This small protein fraction may be sometimes sufficient to provide emulsifying behavior (Dickinson, 2009). More often, the hydrophobic groups in polysaccharides are little and require more quantity to be used for achieving desired emulsification. (Gharsallaoui et al., 2010b; Lim and Roos, 2015).

2.3. Alternative techniques of interface stabilization

Though food emulsifiers have varied properties, no single emulsifier is effective both at producing small oil droplets and at preventing aggregation under different stress conditions (varying pH, ionic strength, freezing and heating). The existing methods of preparing emulsions using conventional emulsifiers suffers several limitations. As an alternative strategy, in the past few years, there is an improved interest in creating emulsions with composite interface (Liu et al., 2016b). Proteins and polysaccharides have an important role to play in the formulation of emulsion interface (Yadav et al., 2010). Emulsions with composite interface can be created by forming multilayer emulsions or by forming protein-polysaccharide conjugates. The biopolymers interact electrostatically at the interface in case of multilayer emulsions. On the other hand, conjugates have a covalent bond joining the biopolymers. These two techniques creates an interface that has the beneficial traits of the individual biopolymers. The principles underlying the preparation of multilayer and conjugate stabilized emulsions are given in figure 2.1.

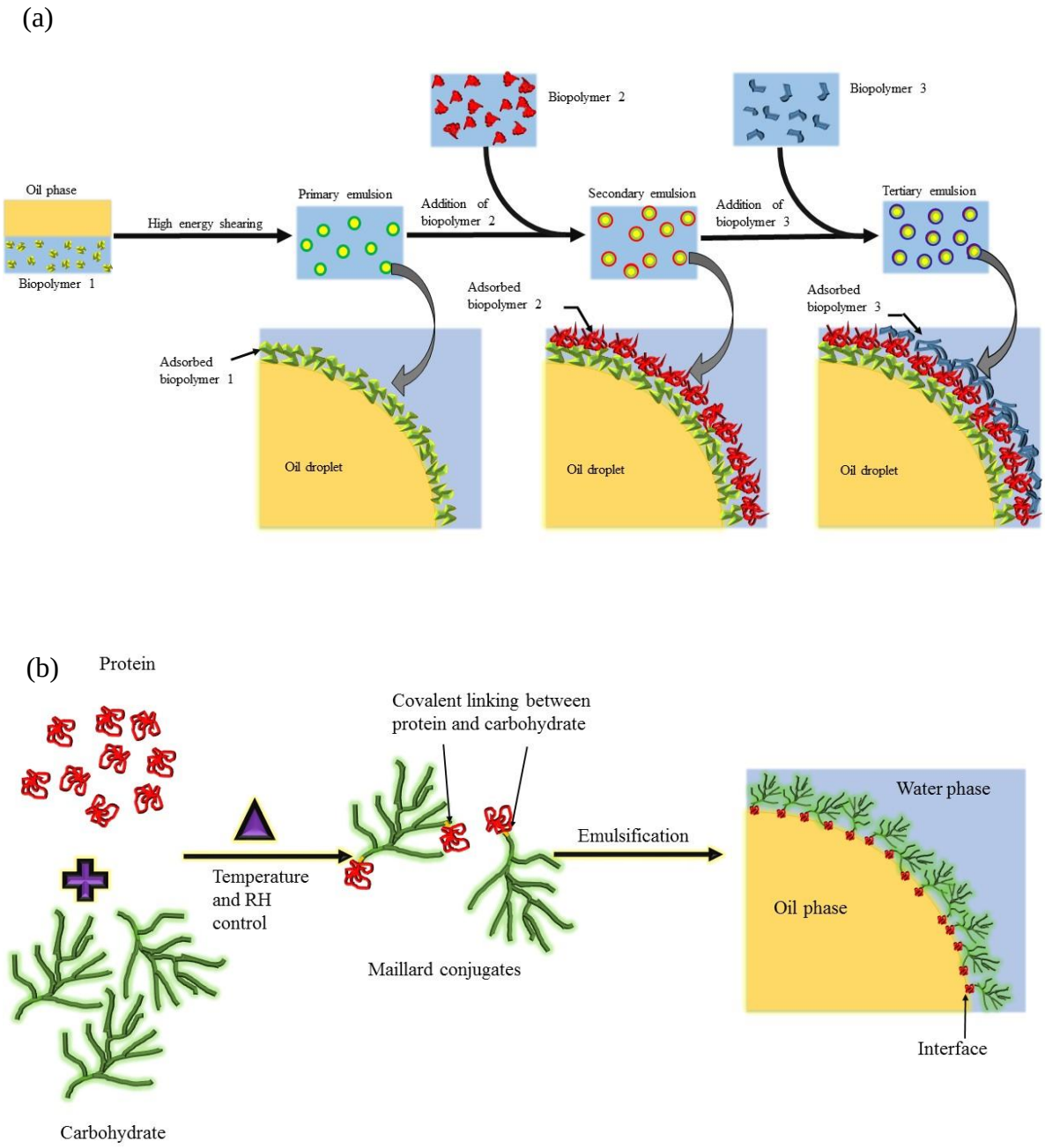


Figure 2.1: (a) Depicts schematically the general stages of multilayer emulsion formation by electrostatic deposition. First, a primary emulsion is prepared by shearing and homogenization of oil and water phases in the presence of emulsifier 1. Secondary emulsions are formed by the subsequent addition of biopolymer 2. Further, tertiary emulsions are prepared by adsorbing biopolymer 3 on the secondary emulsion. (b) Depicts schematically the formation of a protein-polysaccharide conjugate by covalent linking. The arrangement of the Maillard conjugate on the oil/water interface of an emulsion system is shown (Sivapratha and Sarkar, 2016).

Multilayer emulsions under certain circumstances, form emulsions that have superior stability to coagulation over time, variation in salt concentration, temperature and pH (Aoki et al., 2005). This improved ability of secondary and tertiary emulsions are

attributed to modifications in rheology and interfacial thickness (Salminen and Weiss, 2014). Nevertheless, the choice of appropriate biopolymers making up the multilayers are crucial. The successful formulation of multilayered emulsions result from a variety of interdependent factors such as polymer characteristics (concentration, solubility and molecular weight), solution characteristics (pH and ionic strength), droplet features (size, distribution and charge) and mixing conditions (stirring, sonication and sequence of addition) (Pallandre et al., 2007).

2.4. Preparation of multilayer emulsions

A physically stable emulsion is formed by creating a multiple-layered interface by layer-by-layer electrostatic deposition technique. When polyelectrolytes with opposite charge to the electric potential possessed by primary emulsion come into close contact, they interact constructively and form a second layer above the first layer, with charge reversal of the bilayer droplet. The procedure may be repeated with biopolymers of opposite charge to form more layers, depending upon the requirement and feasibility (Wei and Gao, 2016). This method is referred to as layer-by-layer (lbl) deposition approach (Aoki et al., 2005; Liu et al., 2016a; Pallandre et al., 2007; Xiang et al., 2016). The lbl deposition technique enables manufacturers to create interfacial coatings on oil droplets with desirable control over characteristics like layer thickness, environmental sensitivity, permeability and electrical charge (Gudipati et al., 2010). On the other hand, when the biopolymers used for preparing multiple layers amounts to no net charge on the emulsion droplet, electrostatic or steric repulsion may prevail (Gharsallaoui et al., 2010b). In this way, more layers with suitable charge interactions can respond constructively or when interactions are not favorable, respond destructively.

Multilayer emulsions are complex at the interfacial region. The interfacial membrane is made up of more than one material that interact in a way to retain the oil phase dispersed in the aqueous phase of o/w emulsion. The charge possessed by the lipid interface is the dominant factor that facilitates or retards the adherence of subsequent layers. Most biopolymers used as emulsifiers in food industry possess charge, that is, they may be cationic or anionic emulsifiers. In such cases, the charge of the adsorbed emulsifier will be the net charge of the emulsion droplet. There are some non-ionic emulsifiers used for stabilizing emulsions that do not possess their own charge. However, the emulsions droplets stabilized by such emulsifiers will have a net negative charge

contributed by the fatty acids and other lipid components. The role charge plays in forming emulsion is stressed as this is the basis for the subsequent layers to attach on. A polyelectrolyte of charge opposite to the charge of the first layer will adsorb onto it. It is also observed that the magnitude of the charge on the second layer is slightly higher than that is required to counteract the charge on the first layer, thus enabling the deposition of subsequent layers. Though, the first layer requires energy intensive processes like homogenization and sonication, the deposition of subsequent layers on the emulsion interface requires techniques to carefully facilitate electrostatic interaction. For example, change of pH of the aqueous phase that will reverse the charge on protein emulsifier and enable electrostatic interaction with the primary interfacial layer (Guzey and McClements, 2006).

Compared to single-layered emulsion, multilayered emulsions produced by lbl approach enhance stability against changes in ionic strength, pH, thermal processing operations, ageing, drying, lipid oxidation and freeze-thaw cycling (Lim and Roos, 2015). The relatively uncomplicated procedure is a strong tool for food manufacturers to improve resistance against stresses. In addition, multilayered emulsions also create stable dispersions with advantageous physicochemical characteristics derived from synergy of proteins and polysaccharides (Pallandre et al., 2007).

2.5. Introduction to biopolymers used.

The biopolymers used in the current research to form emulsions are sodium caseinate, sodium alginate and chitosan that contribute the primary, secondary and tertiary layers, respectively. These biopolymers are selected as they are natural and are used as food ingredients. In addition, flaxseed oil was the bioactive ingredient that was used as oil phase.

Sodium caseinate is an inexpensive commercial material derived from bovine milk protein, casein. Caseins exist in milk of neutral pH as micelles bound to calcium salts. The replacement of the calcium salts with sodium salts lead to the formation of sodium caseinate. Commercially, sodium caseinate is manufactured by precipitating casein using acid and subsequently, treating with sodium hydroxide. These two steps are followed sequentially in order to acidify whole casein to pH 4.6 and to readjust pH to 6.7. Then, the treated caseins are pasteurized and spray dried. This route is meant to eliminate calcium phosphate that binds the native caseins present in milk. Sodium caseinate has improved

solubility than casein, isoelectric point of 4.5 and average molecular weight of approximately 24 kDa. It has a supramolecular construction composed of αs_1-casein, αs_2-casein, κ-casein and β-casein. Of these, β-casein and αs_1-casein that together constitute 75% of the total milk casein contribute to the impressive emulsifying properties due to their amphiphilic nature (de Figueiredo Furtado et al., 2016; Dickinson, 2010; Xu et al., 2014). Sodium caseinate in emulsions system align rapidly at oil/water interface during homogenization due to the presence of exposed non-polar residues. Sodium caseinate stabilized emulsions exhibit stability over extended periods owing to a combination of steric and electrostatic stabilization mechanisms (Dickinson et al., 2003; Farshchi et al., 2013). Sodium caseinate is a flexible protein that forms an interfacial structure different from those formed by globular proteins like β-lactoglobulin (Pallandre et al., 2007). For instance, Pallandre et al. (2007) stated that more open and thicker interfacial structures were formed by caseinate than β-lactoglobulin.

Sodium alginate is a water soluble sodium salt of alginic acid. Similar, commercially accessible water insoluble salts are alginic acid and calcium alginate and soluble salts are ammonium alginate and potassium alginate. Sodium alginate is a carbohydrate polymer of α-L-glucuronate (G) and (1–4)-linked β-D-mannuronate (M) residues. It is obtained from brown algae such as *Laminiaria digitata* and *Laminiaria hyperboria* and seaweeds. Being anionic in nature, alginate forms complexes with cationic biopolymers. While forming gel, sodium alginate can hold 200 to 300 times its weight of water. Under acidic conditions, alginate is stable but swells and disintegrates in the presence of mild alkali. The popularity of the polysaccharide is derived from its attractive properties such as low toxicity, biocompatibility, mild gelation and crosslinking resulting from addition of divalent cations like Ca^{2+} and availability at reasonable cost. The applications of sodium alginate in food industry is as gelling, bulking, thickening, emulsifying and foaming agent (Lee and Mooney, 2012; Rani et al., 2013; Sarika and James, 2016; Yoo et al., 2006).

Chitosan, a natural antimicrobial polysaccharide is used extensively in the fields of tissue engineering, delivery of drug and functional foods (Li et al., 2016). It forms positively charged polyelectrolyte solution (Liu et al., 2008). Cationic nature is unique to chitosan as most natural polysaccharides are anionic or neutral. It is derived from deacetylation of chitin, which is an abundant polymeric carbohydrate found in shellfish. The amine group (NH_2) group in chitosan picks up a proton in acidic media and forms

NH_3^+, which gives antimicrobial and antifungal property. These cations attract and bind to negatively charged fungal and bacterial cell walls, thus destroying them. In addition, chitosan is compatible with living tissue and is non-toxic. It is hydrophilic in nature. Such interesting features have found chitosan application in several areas including waste water treatment, wound curing, artificial skin fabrication, cosmetics and food preservation (Harish Prashanth and Tharanathan, 2007; Weiss et al., 2006).

2.6. Properties and application of multilayer emulsion system

Secondary and tertiary emulsions are referred to as multilayer emulsions. Multilayer emulsions enable the protection of lipophilic food components that are retained in the oil droplets using a variety of proteins, phospholipids and polysaccharides (Dima et al., 2015). Secondary emulsions when compared to primary emulsions have more stability to freeze-thaw cycling and fluctuations in ionic strength, pH and temperature (Zhao et al., 2015).

Studies have attempted the use of lactoferrin and β-lactoglobulin (β-lg) having isoelectric points of 8.5 and 5.0, respectively, to form laminated coatings on emulsion droplets in order to confer certain functional qualities. The coatings formed from mixed interfacial systems were found to be stable to pH range 3.0 to 7.0, thermal treatment for 20 minutes at 21 to 90 ºC, pH 7 and ionic strength 0 to 500 mM NaCl as well as 0 to 60 mM $CaCl_2$ (Schmelz et al., 2011). The experiments of Mao et al.(2013) indicated that multilayer emulsions having lactoferrin, as the primary or secondary layer reduced the colour fading of protected β-carotene (Mao et al., 2013).

The fields of active compound delivery through food could benefit from the bilayer emulsion system that can protect degradable lipophilic compounds. Emulsions of ω-3 oils enclosed in a double layer protein coating of caseinate and lactoferrin was physically stable and the reactive oil decomposed less into thiobarbituric acid reactive substances and lipid hydroperoxides, which indicate the extent of oxidation. The comparison was made with monolayer caseinate emulsions (Lesmes et al., 2010). Surface charge of multilayered emulsions consisting of primary sodium caseinate and secondary lactoferrin layers had to be sufficiently high for the emulsions to be stable. However, it was observed that the surface charge had little role to play in the protection of vulnerable active compounds in

lipid phase and degradative factors of aqueous phase from interacting with each other (Chaprenet et al., 2014). Hu et al. (2003) also emphasized that apart from the magnitude of droplet charge, underlying factors such as free-radical scavenging of amino acids, interfacial film thickness and chelating property of proteins could affect oxidative stability of lipids.

Bilayer emulsions coated with inner anhydrous milk protein and outer gelatin was found to be consistent at pH 7.0 and unstable at pH values 3.0 and 5.0. The emulsion behavior was studied using scanning electron microscopy (Tippetts et al., 2013). Layer-by-layer deposition of fish gelatin and whey protein isolate (WPI) was used to stabilize and prevent oxidative breakdown of fish oil-in-water emulsions for incorporation into milk and citrus beverage emulsions (Taherian et al., 2011). Such multiple layer coatings could be considered apt for human consumption as they are made from food-grade ingredients and are digested easily. Oil enclosed by lactoferrin/ β-lg or β-lg /lactoferrin nanolaminates were digested easily in simulated gastrointestinal conditions (Schmelz et al., 2011).

Natural polysaccharides occur with varied properties and structure. Their chemical and physical combination (bilayers) were attempted in order to make use of the properties of the constituents (Ramírez-Santiago et al., 2012). Bilayer emulsion of internal cationic calcium phosphate–chitosan composites and outer anionic mesquite gum were fabricated by lbl approach to protect chili oleoresin. Stability against degradation of carotenoids and against aggregation of the prepared oleoresin-in-water emulsion was best when the mass ratio of calcium phosphate–chitosan complex and mesquite gum was 1:10 (García-Márquez et al., 2015).

A primary conventional emulsion stabilized with 10% octenyl succinic anhydride starch (OSA-starch), a modified version of starch where the OSA end is hydrophobic, was studied for its interaction with xanthan gum. Improvement in emulsion stability was associated with thickening droplet interface and increase in viscosity of aqueous phase (Krstonošić et al., 2015). Oleoresin capsicum was formulated as double-layer nanoemulsion by self-assembly using alginate and chitosan interfacial layers to increase stability. The study claimed the size of the nanoemulsion formed to be less than 20 nm (Choi et al., 2011).

Hou et al.(2010) demonstrated that the concentration of secondary layer in multilayer emulsion played an important role in stabilizing β-carotene emulsions

consisting of soybean soluble polysaccharide as primary emulsifier. Below a critical concentration of 0.33% of secondary emulsifier chitosan, droplet size had reached elevated levels indicating aggregation; above 0.33% chitosan concentration, the globule size dropped. The most stable as well as least β-carotene degradable emulsion was obtained when the concentration of chitosan was 0.5% for an oil-in-water emulsion with 10% oil phase. Layer over layer deposition of emulsifiers was concluded from the charge reversal of emulsions from -34 to 58.2 mV, as positively charged chitosan adsorbed on to the soybean soluble polysaccharide stabilized anionic droplets (Hou et al., 2010). Molecular weight of chitosan was also found to influence the stability of carotenoid emulsions (Hou et al., 2012).

The instance of bilayer emulsions prepared using protein and carbohydrate biopolymers are the most common ones as lbl deposition technique requires the two layers to have opposite charges. Proteins and polysaccharides are charged entities in solution. Multilayer interface is contributed by monolayers of different interacting biopolymers. It is to be observed that innermost biopolymer forms monolayer coating and sufficient biopolymer is required to completely cover the surface before the adherence of subsequent layers (Đorđević et al., 2015).

Using lbl deposition technique, bilayer emulsions of thickness 80-170 nm of inner β-conglycinin and outer high methyl pectin layers was formed and its surface morphology was analyzed. Positively charged fish oil-in-water primary dispersion, using β-conglycinin was prepared by high-shear mixing, followed by pectin deposition assisted by homogenization (Xiang et al., 2016). The thickness of the interface could be measured at the submicron level after freeze fracturing the emulsion droplets using cryo-SEM (Humblet-Hua et al., 2012) . However, such bilayer emulsions were not noticed with primary emulsion of whey protein isolate, on which bacterial cellulose was added (Paximada et al., 2016). Therefore, it can be inferred that not all positively charged proteins and negatively charged polysaccharides form an electrical bilayer and there are many more interactions that influence lbl deposition.

Addition of pectin was associated with the formation of multiple interfacial coatings on pea protein isolate stabilized primary emulsions. Secondary emulsions were stable except for bridging flocculation observed at low pH values and low concentration of pectin. The rigidity of interfacial membrane was believed to have increased by pectin

addition (Gharsallaoui et al., 2010b). Sufficient beet pectin was required for complete coverage of the surface of primary emulsion, if not, bridging flocculation was prevalent and the uniformity of the emulsion collapsed. Also high concentration of pectin increased the viscosity, adding up to physical stability of olive oil-in-water emulsions (Kaltsa et al., 2014). The physical stability of bilayer emulsions of sodium caseinate and κ-carrageenan were evaluated at two different pH values 3.5 and 7. At less than 0.25% (w/v) κ-carrageenan concentration, bridging flocculation was observed at both pH values. However, at both the evaluated pH, stable emulsions were obtained at 1% (w/v) polysaccharide coated on a primary emulsion surrounded by 0.5% (w/v) sodium caseinate (Perrechil and Cunha, 2013).

The interaction of microcrystalline cellulose (MCC) with soy protein hydrolysate (SPI) stabilized curcumin emulsion could be viewed as multilayer emulsion as it was observed that the interface became more negatively charged with the interaction with MCC (Xu et al., 2016b). Hydrolysates of SPI were prepared in the test using papain enzyme and was found to be a safe and well accepted method of modification (Chen et al., 2011)

Layer-by-layer approach is the most preferred method by which multiple emulsions are prepared as illustrated by Azarikia and Abbasi (2015). Even when mixed systems of whey protein and tragacanth gum were sonicated, lower apparent viscosity was observed which was associated with bridging flocculation on sonication. When emulsions with same combination of emulsifiers was prepared by lbl method stable emulsions were obtained. *Astragalus* is an Asiatic species from which tragacanth gum is obtained. The hydrocolloid is best known for its suitability for acidic emulsions (Abdolmaleki et al., 2016).

Multilayered emulsions were believed apt for delivery of active component through human food. At low pH, flocculated sunflower oil-in-water emulsions deposited with coatings of internal WPI and external gum Arabic was found to be stable and the system destabilized at higher pH values that is, deflocculated into individual oil particles. Such variations in pH could be expected in human alimentary canal where active component is to be protected at the low pH of stomach and enhance the absorption of active component in intestine (Lim and Roos, 2015).

Enzymatic cross-linking of the secondary pectin layer formed a coating that was associated with superior physical stability in the occurrence of deleterious factors. The primary layer surrounding the oil droplets present dispersed in the aqueous phase was whey protein isolate. Beet pectin was facilitated to adsorb on to the primary emulsion by pH adjustments to form secondary emulsions. Subsequently, the enzyme horseradish peroxidase was added to induce cross-links among pectin molecules (Li et al., 2012). Enzymatically cross linked multilayer emulsions have been attributed to various functional properties. For instance, emulsified oils were prepared to reduce the absorption of fats in gastrointestinal tract (GIT) as an attempt to combat obesity. The release of fatty acids from the emulsion was studied using a simulated GIT containing mouth, gastric and intestinal phases. The cross linked gelatin-beet pectin multilayered emulsions, where covalent bonding was induced by using laccase enzyme were found to be slightly stable in upper gut but were digested in simulated small intestine (Zeeb et al., 2015).

Probiotics can improve immune system of humans. Due to its numerous health benefits, consumption of live beneficial microbes has gained importance. The main hindrance in incorporating probiotics in food is that it is labile to destruction during processing and the harsh environment of the stomach. The organisms have to inhabit the large intestine after gastrointestinal transit (Sathyabama and Vijayabharathi, 2014). Spray dried *Lactobacillus salivarius* NRRL B-30514 was suspended in anhydrous milk fat and formed into oil-in-water emulsion. The emulsion formed here had a primary layer of sodium caseinate or whey protein, followed by electrostatic deposition of secondary layer pectin. Such emulsions were found to keep the cells viable (Zhang et al., 2015).

Polyunsaturated fatty acids are vulnerable to oxidative degradation due to large number of double bonds present in them and suitable emulsion interface is believed to form a protective barrier against rancidity. Conventionally, antioxidants and chelators are used for protection of lipids in emulsion system. The manipulation of interface to form a dense and physically thick layer around the oil globule, with an overall positive charge is believed to pose a barrier that separates pro-oxidants and lipid hydroperoxides (Scheffler et al., 2009). The segregation between the metal ions and oxygen in the water phase and peroxides in the oil phase, prevents the progress of further chemical reaction leading to degradation products (Gharibzahedi et al., 2013).

Secondary emulsion of inner pea protein and outer pectin layers were subjected to dry processing and showed that stability was ensured using size, charge, microstructure and creaming measurements (Gharsallaoui et al., 2010a). In another study, spray dried secondary emulsions consisting of pea protein isolate and pectin had produced emulsions with higher resistance to oxidative breakdown of polyunsaturated lipids (Aberkane et al., 2014). W/o/w double emulsions were fabricated to protect saffron extract by making use of whey protein concentrate and pectin double layers and then, dried. Saffron extract finds application in food and pharmaceutical industries for its antioxidant property, natural colour and therapeutic value (Esfanjani et al., 2015).

The lbl coated formulations suppressed the loss of oil-soluble bioactives such as carotenoids (Lim and Roos, 2016). Lactoferrin stabilized orange oil-in-water primary emulsions were made into bilayer emulsions by coating with two polysaccharides - soybean soluble polysaccharide and beet pectin (one at a time) in an attempt to protect the orange oil from oxidation and the bilayer emulsions were compared. The oil consisted of volatile compounds including octanal, decanal, limonene and geranial, prone to oxidation. Bilayers consisting of internal lactoferrin and beet pectin had protected the volatile compounds better (Zhao et al., 2015). Xiang et al. (2015) also studied the encapsulation of citral emulsions using milk proteins (lactoferrin, β-lactoglobulin or α- lactalbumin) and beet pectin with the bilayer emulsions consisting of internal lactoferrin and external beet pectin exhibiting the best protection of neral and geranial volatile compounds from oxidative degradation during storage at 25 ºC.

Emulsions with three-layer interface are referred to as tertiary emulsions. The concentration of emulsifiers that form multilayered emulsion has to be carefully monitored so that depletion flocculation and bridging flocculation is prevented and amount of unadsorbed emulsifier is reduced. Triple layer emulsions were formed using emulsifiers - soy protein isolate-OSA-starch-chitosan by lbl deposition and compared with secondary emulsions of soy protein isolate-OSA-starch layers and primary emulsion prepared from soy protein isolate monolayer. The superiority among primary, secondary and tertiary emulsions were established on the basis of ζ-potential, microstructure analysis and diameter of droplet in response to varying pH, thermal treatment and salt concentration. The results indicated that tertiary emulsions had a better steric stabilization and protected the integrity of emulsion from stress factors that are presumed to be a part of aqueous phase (Noshad et al., 2016). Other multiple layer emulsion membrane produced by

electrostatic interaction that protected the oil droplets from environmental stresses include sodium dodecyl sulfate-chitosan-pectin membranes and β-lactoglobulin-ι-carrageenan-gelatin membranes (Aoki et al., 2005; Gu et al., 2005).

Aoki et al. (2005) prepared a trilayer emulsion using corn oil and an interfacial membrane system consisting of sodium dodecyl sulphate-chitosan-pectin. They also compared the anionic primary, cationic secondary and anionic tertiary emulsions for their resistance to environmental stress factors including freeze-thaw cycling, increasing ionic strength and varying pH. Tertiary droplets had good stability among the compared emulsions. Gu et al. (2005) while studying the effect of temperature and added salt on the stability primary (5% w/w corn oil, 0.5% w/w β-lactoglobulin), secondary (5% w/w corn oil, 0.5% w/w β-lactoglobulin+ 0.1% w/w ι-carrageenan) and tertiary (5% w/w corn oil, 0.5% w/w β-lactoglobulin+ 0.1% w/w ι-carrageenan+ 0.6% w/w gelatin) emulsions at pH 6.0 stated that in the absence of added salt, ζ-potential was independent of rise in holding temperature. However, in the presence of 150 mM added salt, primary and tertiary emulsions became more negative while secondary emulsions had unaltered ζ-potential.

The buffer solutions that are used to maintain pH during emulsion preparation was found to influence the emulsion stability in triple layered emulsion systems consisting of a combination of whey protein isolate, pectin and fish gelatin. It was noticed that citrate buffer was unsuitable for making up the aqueous phase of multilayer emulsion and that its ionic composition played a key role in maintaining the emulsified globules in dispersed form (Zeeb et al., 2013). Noshad et al.(2015) showed that triple layer emulsions were superior to freeze-thaw treatment than bilayer or monolayer o/w emulsions which was accredited to the thick interface. When the water phase was frozen, primary and secondary emulsions exhibited droplet flocculation while, tri-layer emulsions did not.

Lutein emulsions were protected using a stable triple layer interfacial membrane made up of whey protein isolate-flaxseed gum-chitosan which exhibited low lutein degradation than secondary and primary emulsions. Chitosan had to be added at a minimum level of 1% for a stable oil-in-water emulsion at pH 3.0 containing 10% medium chain triacylglycerol oil into which lutein was incorporated (Xu et al., 2016a). Jo et al.(2015) attempted to produce stable triple layer fish oil-in-water emulsion that reduced the off flavours due to oxidation of oil. Also *trans*-cinnamaldehyde was included into the oil phase so as to mask the undesirable odour. It was noticed that as the number of interfacial

layers increased, oxidative stability increased. Fish oil release was prevented better with primary 1.25% Tween 20 layer, secondary 0.1% chitosan layer and tertiary 0.2% low methoxyl pectin layer combination.

Comparative oxidative stability of primary, secondary and tertiary emulsions were studied by Gudipati et al. (2010) in fish oil-in-water emulsions made up of citrem, chitosan and sodium alginate as interfacial materials. They reported that cationic secondary (citrem-chitosan) emulsion were more stable to lipid oxidation than primary and secondary emulsions. This research also studied the lipid digestibility using in-vitro digestion model simulating intestinal environment.

2.7. Understanding the theory behind oxidation of lipids

One of the applications of multilayer emulsion system is the protection of vulnerable PUFAs. General oxidation of unsaturated lipids has been studied by several researchers (Frankel, 1984; Frankel, 1991; Laguerre et al., 2007; Schaich, 2005). The autoxidation of lipid to yield monohydroperoxides is conveniently studied as three distinct stages- initiation, propagation and termination.

2.7.1. Initiation

The reaction between unsaturated lipid (LH) and oxygen (O_2) cannot proceed spontaneously as the activation energy barrier is very high. Therefore, the direct reaction as in equation 1 is not possible. The reason behind high activation energy is that the spin directions of ground state lipid and oxygen are opposite to each other. External physical agents such as ionizing radiation, heat, UV light and internal chemical agents like free radicals, metal ions and metalloproteins can serve to initiate lipid oxidation (Laguerre et al., 2007). Therefore, the activation energy barrier could be overcome when oxygen is present as chemically labile reactive oxygen species such as singlet oxygen ($^1O_2$) and hydroxyl radical (HO•) (Berton-Carabin et al., 2014) as well as lipid peroxyl (LOO•) and lipid alkoxyl (LO•) radicals. On reaction with lipids, hydroxyl radicals have the greatest reaction rates (Laguerre et al., 2007). Laguerre et al. (2007) argued that the probability of existence of more than one initiation step has made initiation mechanism challenging to comprehend.

$$\mathbf{LH + O_2 \rightarrow LOOH} \qquad \text{..... 1}$$

Lipid autoxidation starts with the breakage of hydrogen atom from an allylic site relative to unsaturation point of fatty acid. This yields an alkyl free radical (L•) as in equation 2. A chemical species with unpaired electron is called a free radical. Being an unstable intermediate, it abstracts hydrogen from other chemical species such as lipids. During this phase, oxygen consumption will be low. At the end of initiation stage, oxidation abruptly accelerates and peroxide concentration rises substantially (Laguerre et al., 2007).

$$\mathbf{LH \xrightarrow{initiator} H + L\bullet} \qquad \text{......................... 2}$$

The formation of alkyl free radical (L•) or lipoyl free radical from unsaturated lipid (LH) can occur through different pathways as follows.

i. Direct reaction of transition metal (M) ions and LH.

$$\mathbf{LH + M^{3+} \rightarrow L\bullet + H^{+} + M^{2+}} \qquad \text{......................... 3}$$

ii. Reaction with oxygen radicals formed from metal autoxidation.

$$\mathbf{Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-}\bullet \leftrightarrow HOO\bullet + H^{+} \rightarrow L\bullet + H_2O_2} \qquad \text{......................... 4}$$

$$\mathbf{(2O_2^{-}\bullet)or\ (O_2^{-}\bullet\ and\ HOO\bullet) \rightarrow H_2O_2 + O_2} \qquad \text{......................... 5}$$

$$\mathbf{H_2O_2 + Fe^{2+} \rightarrow HO\bullet + OH^{-} + Fe^{3+}} \qquad \text{......................... 6}$$

$$\mathbf{HO\bullet + LH \rightarrow H_2O + L\bullet} \qquad \text{......................... 7}$$

iii. Lipid peroxides (LOOH) present in oil in trace quantities can undergo chemical changes such as reduction, decomposition and oxidation resulting in lipid free radicals. Metal catalyzed oxidation and reduction reactions of peroxides are given as:

$$\mathbf{LOOH + M^{2+} \rightarrow LO\bullet + OH^{-} + M^{3+}} \qquad \text{......................... 8}$$

$$\mathbf{LOOH + M^{3+} \rightarrow LOO\bullet + H^{+} + M^{2+}} \qquad \text{......................... 9}$$

These alkoxyl and peroxyl radicals participate in initial stages of lipid oxidation.

iv. Decomposition of hydroperoxide by heat.

$$\mathbf{LOOH \rightarrow LO\bullet + HO\bullet} \qquad \text{..................... 10}$$

While considering the kinetics of reaction 10, the activation energy barrier is high. This reaction can alternatively proceed favorably through another pathway using two lipid hydroperoxides as indicated in equation 11.

$$\mathbf{2LOOH \rightarrow LO \bullet + H_2O + LOO \bullet} \quad \ldots\ldots\ldots\ldots\ldots\ldots 11$$

2.7.2. Propagation

The major species developed in the initiation stage through various pathways is alkyl free radical (L•). In the propagation stage, L • reacts readily with molecular oxygen to yield peroxide radical (equation 12).

$$\mathbf{L \bullet + O_2 \rightarrow LOO \bullet} \quad \ldots\ldots\ldots\ldots\ldots\ldots 12$$

Being highly unstable, the peroxide free radicals abstract hydrogen from other lipid molecules and generate a different set of free radicals.

$$\mathbf{LOO \bullet + L'H \rightarrow LOOH + L' \bullet} \quad \ldots\ldots\ldots\ldots\ldots\ldots 13$$

In the reactions indicated by equations 12 and 13, alkyl free radical (L•) participates as a reactant and another alkyl free radical (L'•) is produced as a product. The new alkyl free radical (L'•) generated attacks another oxygen molecule and the chain reaction goes on. Therefore, alkyl free radical is regenerated in the propagation reaction with the formation of lipid hydroperoxides as byproduct and consumption of molecular oxygen.

The primary products of lipid oxidation are at all times monohydroperoxides commonly referred to as lipid hydroperoxides (LOOH). The number of monohydroperoxides formed from a PUFA molecule depends on the number of unsaturation sites it holds and is given by 2n-2. For instance, linoleic acid (18:2), linolenic acid (18:3), arachidonic acid (20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (20:6) give rise to two, four, six, eight and ten (Esterbauer, 1993). These findings explain the high rates of oxidation of fats with increased unsaturation sites.

Oxygen uptake is a measure of lipid oxidation. The reason for measurement of oxygen uptake of oil containing sample placed in a closed vessel is that oxygen is depleted as indicated in equation 12 and 13. Though it is not a sensitive and reliable method for estimating lipid oxidation, it is used alternatively (Berton-Carabin et al., 2013; Berton et al., 2011; Villière and Genot, 2006). The rate of formation of lipid hydroperoxides

accelerates substantially as a result of chain reaction of the radicals. The primary products of lipid oxidation are usually estimated by measuring the amount of hydroperoxides often conveyed as peroxide value using spectroscopic techniques (Kargar et al., 2011; Liu et al., 2015a; Qiu et al., 2015b). The quantity of detected hydroperoxide over time will give an estimate about the amount of peroxides created by the consumption of fat as well as the quantity depleted into secondary oxidation products. Estimation of conjugated dienes also measures the extent of formation of primary oxidation products (Dridi et al., 2016). The above discussed reaction scheme is the simplified reaction pathway referred to as classical route. Practically, there are a lot of sophisticated reactions taking place as discussed by Schaich (2005).

According to the classical route, the lipid hydroperoxides formed undergo decomposition through non-radical and radical pathways to yield secondary products of lipid oxidation. The most important pathway being the hemolytic scission at the double bond adjacent to hydroperoxyl group. These reactions are promoted by heat and presence of metals (Berton-Carabin et al., 2014). They can undergo homolytic cleavage at LO-OH forming alkoxyl free radical (L”O•), which in turn undergo cleavage at the C-C giving rise to a several compounds- aldehydes, ketones, esters, furans, hydrocarbons, alcohols and lactones. Another pathway may be taken by hydroperoxide, where it reacts again with oxygen to form ketohydroperoxides, epoxyhydroperoxides, bicyclic endoperoxides, cyclic peroxides and dihydroperoxides. Similar to monohydroperoxides, the other peroxides can also decompose to form volatile products. Yet another means the hydroperoxide can form volatile breakdown products is by the degradation of condensed dimers and polymers of hydroperoxides (Frankel, 1984). The cleavage and decomposition of peroxides give a myriad of low molecular weight compounds of which, the volatile compounds are the cause of off-flavour associated with lipid degradation.

In addition to the estimation of hydroperoxide or conjugated diene concentration, it is a common practice to measure a minimum of one marker of secondary oxidation product. The widely used techniques to estimate secondary products of lipid oxidation are TBARS (thiobarbituric acid-reactive substances) assay, assessment of ρ-anisidine value and volatile compounds. TBARS assay uses spectroscopy to test and quantify the presence of various compounds markedly malonaldehyde (Alfaro et al., 2016; Scheffler et al., 2009; Zhou and Elias, 2013). ρ-anisidine value also uses spectroscopy to determine the concentration of total aldehydes (Julio et al., 2015; Kargar et al., 2011). Volatile

compounds resulting from lipid oxidation such as hexanal and propanal are measured using dynamic or static headspace gas chromatography (Hu et al., 2003; Taherian et al., 2011; Zou and Akoh, 2015)

2.7.3. Termination

The radicals formed during the propagation phase react together and form non-radical materials during termination stage. These non-radical products are stable and no further oxidation chain reaction occurs. Due to varied pathways of radical formation, many products are formed and have not been extensively elucidated (Berton-Carabin et al., 2014).

$$\mathbf{LOO\bullet + LOO\bullet \rightarrow nonradical\ products} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ 14$$

The termination could be brought by deliberate addition of antioxidant compounds. Antioxidants form non-radical products or stable radicals (A•) by reacting with peroxide radicals.

$$\mathbf{LOO\bullet + AH \leftrightarrow LOOH + A\bullet} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ 15$$

$$\mathbf{A\bullet + LOO\bullet \rightarrow nonradical\ products} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ 16$$

$$\mathbf{A\bullet + A\bullet \rightarrow nonradical\ products} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ 17$$

The critical step in oxidation is the formation of highly reactive alkoxyl and peroxyl radicals at interface from lipid hydroperoxides due to its reaction with transition metals. The main factor promoting lipid oxidation is the transition metals present in aqueous phase. Iron is believed to be the most influential transition metal as specific iron binding proteins for instance, lactoferrin and transferrin had strongly inhibited lipid oxidation (Huang et al., 1999; Mancuso et al., 1999). In addition, the occurrence of iron reducing agents can accelerate oxidation because ferrous being highly soluble and reactive is a more capable prooxidant as compared to ferric ion (Waraho et al., 2011). Among other factors influencing lipid oxidation in emulsions are addition of iron salts, hydrophilic or lipophilic radical initiators and heme proteins, storage temperature and availability of oxygen (Berton-Carabin et al., 2014).

2.8. Theory behind supposed oxidative stability of multilayer emulsions.

Interfacial region of emulsions is a critical region for the progress of oxidation. Emulsions, in general have an oxidation mechanism different from that of bulk oils. This is due to the fact that bulk oils do not have an aqueous phase while emulsions include an aqueous phase that holds pro-oxidants as well as antioxidants. In addition, there is an interface in emulsions between the oil and water where the materials dispersed in both the phases come together and react (Frankel et al., 1994). Researchers believe that transition metals are the primary causative agents of lipid oxidation (Dimakou et al., 2007; Mancuso et al., 2000). This concept is supported by the observation that the use of metal chelator, EDTA as antioxidant in commercial emulsions is enormously efficient (Jacobsen et al., 2001). Waraho et al. (2011) puts forth that interfacial area, composition, charge, thickness and permeability of the interface affect lipid oxidation. Furthermore, presence of heat, light, pro-oxidants, antioxidants and molecular structure of lipids influence oxidation in lipids (Coupland and McClements, 1996; Kargar et al., 2011; McClements and Decker, 2000).

Interfacial area by itself does not affect lipid oxidation rates immensely. The negligible difference in oxidation rates among emulsion varying from 0.2 to 100 μm is because emulsions itself have enormously large interfacial area. Therefore, interfacial area difference among small and large sized droplets is insignificant from oxidative perspective (Waraho et al., 2011). This observation is supported by the experiments of Lethuaut et al. (2002), who demonstrated that lipid oxidation products pentane and hexanal were produced at rates independent of the emulsion interfacial area. The study performed on 30% stripped sunflower oil droplets coated with bovine serum albumin emphasized that emulsification had induced lipid oxidation as compared to non-emulsified controls.

The interface composition could play a pivotal role in oxidation of lipids. A broad variety of substrates may be present in the interface. It includes emulsifiers, minor surface active materials, lipid components like triacylglycerol hydrolysis products, sterols and antioxidants. The interface, being the meeting area of oil and water phases brings into contact pro-oxidants of water phase and oxidizable lipid substrates into close contact (Waraho et al., 2011). Qiu et al. (2015b) deduces that adsorbed protein on interface could

scavenge free radicals, chelate prooxidants metals and form an effective steric barrier, thus retarding oxidation of PUFAs.

Emulsions could have anionic, cationic or neutral surface charge depending upon the emulsifier, pH of the aqueous phase that in turn decide the charge on the biopolymer or other charged entities that could compose or adsorb on to the interface. Several studies have put forth that cationic droplets could repel away the positively charged transition metal ions, thus reducing oxidation (Boon et al., 2008; Hu et al., 2003; Silvestre et al., 2000). For instance, protein stabilized emulsions at pH less than the isoelectric point (pI), formed cationic droplets and thus, prevented lipid oxidation. On the other hand, anionic emulsion droplets of the same protein at pH above pI, detected higher oxidation products (Djordjevic et al., 2008). Experiments of Yi et al. (2016) also supported the theory that emulsions with cationic interfaces had better oxidative stability than neutral or anionic interfaces.

Oil/water interface thickness could have a substantial role in preventing lipid oxidation in emulsions. The presence of the major prooxidant iron in emulsion system is inevitable. It occurs in water, packaging materials and certain food ingredients. Recent approach to prevent lipid oxidation in emulsion system is by physically segregating the aqueous phase prooxidants (specifically iron) and oil phase that harbours lipid peroxides (Kargar et al., 2011). A thick interface can prevent the interaction between oil phase oxidizable materials and aqueous phase pro-oxidants by physically separating them. The interface thickness is contributed by the size of emulsifier molecules and the conformation of hydrophobic and hydrophilic ends at the oil/water interface. Additionally in multilayer emulsion, the thickness of the surface layer is decided by the number of biopolymers that constitute the interface (Djordjevic et al., 2007; McClements and Decker, 2000; Xiang et al., 2015) . Further evidence is obtained from the research of Klinkesorn et al. (2005) that showed that double layer thick interface of lecithin-chitosan coated tuna oil-in-water emulsions prevented chemical degradation of oil than thin interface of lecithin alone.

Similarly, permeability of the interfacial layer was found to impact lipid oxidation because non-permeable interfacial membrane limited the contact between substrates across lipid and water phases. Secondary and tertiary emulsions form a complex and thick barrier (Waraho et al., 2011). It has been found that surfactants protected emulsified oil better than proteins. Therefore, structural homogeneity of the interfacial membrane could be a

key factor in retarding lipid oxidation as surfactants covered the surface uniformly (Berton-Carabin et al., 2013).

From the literature survey, it is hypothesized that multilayer emulsions could be utilized to protect the oxidative degradation of flaxseed oil. This work attempts to use secondary and tertiary emulsions as a vehicle to protect linolenic acid-rich flaxseed oil.

Chapter 3

Experimental methods

3.1. Materials

Chitosan from shrimp shells (Degree of deacetylation: ≥ 75%), sodium caseinate and sodium alginate were procured from Himedia Laboratories Private Limited (Mumbai, India), Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Loba Chemie (Mumbai, India) respectively. Flaxseed oil was a gift from Kamani Oil Industries Private Limited, Mumbai, India and was used without additional processing. Analytical grade acetic acid glacial, sodium chloride, trichloroacetic acid, sodium hydroxide, 1-butanol, ammonium thiocyanate, ferrous sulphate heptahydrate, barium chloride, thiobarbituric acid, butylated hydroxytoluene and laboratory grade hydrochloric acid, sulphuric acid, methanol, 2-propanol, chloroform were obtained from Himedia Laboratories Private Limited (Mumbai, India). 1,1,3,3- tetramethoxypropane was purchased from Himedia laboratories. Deionized water (Milli Q) was utilized for preparation of all samples and solutions.

3.2. Solution preparation

Acetate buffer was obtained by preparing a 5 mM sodium acetate solution in deionized water and subsequently adjusting pH using glacial acetic acid or 1 M sodium hydroxide. Sodium caseinate and chitosan were dispersed in 5 mM acetate buffer at pH 3.0, separately. On the other hand, sodium alginate was dissolved in acetate buffer at pH 4.0. All the biopolymer solutions were stored overnight for thorough hydration. 0.01% (w/v) sodium azide was added to biopolymer solutions to prevent proliferation of microbes.

3.3. Emulsion preparation

Sodium caseinate oil-in-water primary emulsions were formed using a 0.4% (w/v) sodium caseinate emulsifier in buffer solution as continuous phase and 1% (w/v) flaxseed oil as the dispersed phase. The two phases were blended first in a blender to give a course emulsion, which was made finer by homogenizing using an ultrasonicator (QSonica 700, Newtown, CT, USA) at 50% amplitude for a duration of 5 minutes with a duty cycle of 0.5. The sonotrode probe of 12 mm tip diameter vibrated at a frequency of 20 kHz. The final primary emulsion had 1% (w/v) oil and 0.4% (w/v) sodium caseinate.

Sodium caseinate-sodium alginate oil-in-water secondary emulsions were prepared in two steps by using layer-by-layer electrostatic deposition technique. First, a monolayer emulsion of 0.8% (w/v) sodium caseinate and 2 % (w/v) flaxseed oil was prepared. Then, this monolayer emulsion was mixed to 0.5% (w/v) sodium alginate with constant stirring, followed by sonication for 1 minute at 70% amplitude and 0.5 duty cycle. The two preparations, emulsion and alginate solution were mixed in equal volumes for secondary emulsion formation. Therefore, the resulting secondary emulsions had 1 % oil and a bilayer made up of internal sodium caseinate (0.4% w/v) and external sodium alginate (0.25% w/v).

Tertiary emulsion of sodium caseinate-sodium alginate-chitosan was formed by adding a layer of chitosan above secondary layer by lbl deposition. First, a monolayer emulsion of 1.6% (w/v) sodium caseinate and 4 % (w/v) flaxseed oil was prepared. Then, this monolayer emulsion was added to 1.0% (w/v) sodium alginate with constant stirring, followed by sonication for 1 minute at 70% amplitude and 0.5 duty cycle. The resulting secondary emulsions had 2% oil and a bilayer made up of internal sodium caseinate (0.8% w/v) and external sodium alginate (0.5% w/v). This was further diluted with equal volume of chitosan solutions to form tertiary emulsions. The chitosan concentration in the final layer was varied as tabulated in table 2.1 in order to form a stable tertiary emulsion. The interfacial membrane of tertiary emulsions had three layers consisting of different biopolymers. Then the samples were sonicated for 1 minute at 70% amplitude to obtain tertiary emulsions. The pH of the final primary, secondary and tertiary emulsions were not adjusted.

Table 3.1: Concentration of biopolymers making up the layers of tertiary emulsions. Solutions were prepared such that the final concentration of the tertiary emulsion is as tabulated.

Internal layer concentration % w/v	Middle layer concentration % w/v	Outermost layer concentration % w/v
0.4	0.25	0.00
0.4	0.25	0.05
0.4	0.25	0.10
0.4	0.25	0.15
0.4	0.25	0.20
0.4	0.25	0.25
0.4	0.25	0.30
0.4	0.25	0.35
0.4	0.25	0.40

3.4. Storage and sampling

The prepared emulsions were sampled immediately after homogenization or post stress treatment for the analysis of particle size, ζ-potential, creaming stability and initial thiobarbituric acid reactive substances (TBARS) and peroxide value. In addition, the emulsions were transferred to capped polypropylene tubes stored upright at 30 °C in an incubator (LabTech, India LIB-060M) and aliquots were drawn after 1, 2, 3, 4, 5, 6 and 7 days for oxidative studies.

3.5. Emulsion droplet size analysis

The particle size of the emulsion samples were given by Z-Average size. It is the intensity weighted mean hydrodynamic size of a conglomeration of oil particles dispersed in aqueous phase. The emulsions were analyzed for size by Dynamic Light Scattering (DLS) using Zetasizer Nano, Malvern Instruments. Instrument operates with laser at a wavelength of 632.8 nm. The device uses the Stokes–Einstein Equation to correlate the size to Brownian movement of particles. Brownian movement of particles are measured and size is determined under the hypothesis that the larger particles have slower movement (Csaba et al., 2009; Leong et al., 2009). Particle size is evaluated using this theory under the assumption that the particles are homogeneous and spherical. Flocculated and aggregated emulsions are non-spherical and heterogeneous. Data obtained on flocculated

and aggregated emulsion samples must be considered with caution as they do not abide by the assumptions (Aoki et al., 2005). The emulsions were diluted to 0.01% oil prior to particle size measurement. The measurements were taken at a fixed angle of 90°, temperature of 25 °C and refractive indices of acetate buffer and flaxseed oil were taken as 1.33 and 1.48. The measurements were done on three fresh samples and the results were expressed as mean and standard deviation.

3.6. ζ-potential measurements

The velocity and direction of movement of droplets in a well-defined electric field determines the basis of its charge indicated by ζ-potential (Aoki et al., 2005). Particle electrophoresis instrument, Zetasizer Nano ZS, Malvern Instrument, UK was used to measure ζ-potential of diluted emulsion samples. (Noshad et al., 2016). The instrument measures electrophoretic mobility and utilizes Smoluchowski equation to evaluate ζ-potential (Liu et al., 2016a). The emulsions were diluted 100 fold with buffer of appropriate pH in order to prevent multiple scattering effect.

3.7. Optical Microscopy

Emulsions prepared in the study were observed for their structure using a phase contrast microscope (Zeiss Primo Vert, Germany). The samples were mixed thoroughly and a drop of emulsion was placed on a clean glass slide and viewed. The images were acquired by means of Zen 2012 Lite software from the microscope.

3.8. Physical stability measurements

3.8.1. Turbidity

3.5 mL of diluted emulsion samples (0.005 % w/v oil) were taken in plastic spectrophotometer cuvettes of 1 cm path length. The diluted emulsions were left undisturbed at room temperature for 24 hours. Thereafter, turbidity of the samples were recorded at 600 nm wavelength using a UV-visible spectrophotometer. The light beam passed through the cuvette at 10 mm above cuvette bottom, approximately, 30 % of the height of emulsion contained in cuvette. The oil droplets relocated to the top due to creaming or settled at the bottom or sides due to sedimentation or coagulation. These destabilization phenomena resulted in a clear droplet depleted serum at the point where the light passed thus reducing the turbidity. Therefore, a higher turbidity was an indication of better stability and vice-versa (Aoki et al., 2005).

3.8.2. Creaming stability

Fresh emulsions (10 mL) were taken in cylindrical glass vials, tightly sealed and stored undisturbed at room temperature for 7 days. The height of separated cream layers were measured and recorded by means of a ruler.

3.9. Environmental stresses of emulsions

The effect of added NaCl and thermal processing on the particle size and ζ-potential of primary, secondary and tertiary emulsions were investigated as follows:

3.9.1. Thermal stability

To evaluate the thermal stability of emulsions, 5 mL was taken in a 15 mL capped Falcon tubes and subjected to 30 to 90 °C in a temperature controlled water bath (RSB-12, Remi Elektrotechnik limited, India). The incubation was maintained for 30 minutes after which, the emulsions were cooled to room temperature immediately. Then the samples were kept at room temperature until analysis.

3.9.2. NaCl stability

Sodium chloride was added to the primary, secondary and tertiary emulsion samples in powdered state with mixing so as to make a concentration of 30, 50 and 70 mM.

3.10. Oxidative stability

Oxidative stability of primary, secondary and tertiary emulsions of flaxseed oil was assessed by detecting the concentration of lipid hydroperoxide and thiobarbituric acid reactive substances. Hydroperoxide is an intermediate product of lipid oxidation, while TBARS gives an estimate of the final products of oxidation.

3.10.1. Measurement of lipid hydroperoxide

The concentration of lipid hydroperoxide was measured to estimate the progress of lipid oxidation using the methods followed by Qiu et al. (2015b) and Scheffler et al. (2009). Extraction of hydroperoxide from emulsion was carried out by the addition of 0.2 mL of emulsion to 1.5 mL of chloroform: methanol (2:1 v/v) mixture. The mixture was vortexed 3 times for 30 s followed by centrifugation at 1300 rpm for 5 min. 0.2 mL of lower extract layer after centrifugation was drawn out carefully and added to a Falcon tube containing 2.8 mL of methanol: 1-butanol (2:1 v/v) mixture. Then, this 3 mL mixture was

vortexed, followed by the addition of 15 µL of 3.94 M ammonium thiocyanate solution and 15 µL of ferrous iron solution. The reaction mixture was vortexed and allowed an incubation time of 20 minutes prior to the measurement of absorbance at 510 nm.

Ferrous iron solution in the above protocol was formulated by combining two separate preparations. First, 2g of ferrous sulphate heptahydrate was added to 50 mL deionized water and dissolved. Second, 1.6 g of barium chloride was mixed with another 50 mL of deionized water. $BaCl_2$ solution and $FeSO_4$ solutions were mixed together with the addition of 2 mL of 10 N hydrochloric acid (HCl). The precipitate ($BaSO_4$) formed was discarded and the supernatant was preserved at 4 °C in dark for further use.

For the estimation of hydroperoxide, ferric ion standard curve was employed. To prepare a standard curve of ferric ion, first, 0.5 g of iron powder was weighed and reacted with 50 mL of 10 M HCl and 2 mL of 30% hydrogen peroxide (H_2O_2) in a heated water bath for 5 minutes. The iron powder completely dissolved in to the HCl- H_2O_2 mixture. After sufficient cooling, the mixture was made up to 500 mL with deionized water resulting in a ferric ion standard solution of 1000 µg/mL. Further dilutions were made using 1 M HCl to obtain 125, 250, 500 and 750 µg/mL. The standard curve was plotted with concentration in the x-axis and absorbance in the y-axis.

3.10.2. Measurement of Thiobarbituric acid reactive substances (TBARS)

First, thiobarbituric acid (TBA) solution was prepared by dissolving 0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid and 1.76 mL of 12 M HCl in 82.9 mL deionized water. Then, butylated hydroxytoluene (BHT) solution was prepared in ethanol at a concentration of 2% (w/v). Then, the two solutions were combined by adding 3 mL of BHT solution to 100 mL of TBA solution with constant stirring to obtain a TBA-BHT solution.

For spectroscopic analysis, 1.6 mL of emulsion was added to 3.2 mL of TBA-BHT solution taken in a 15 mL centrifuge tube and vortexed for 30 s. This mixture was heated for 15 mins in a water bath and cooled subsequently to room temperature. Sediments were separated by centrifuging the cooled tubes at 12,000 rpm for 10 min. The supernatant was transferred to the cuvettes and absorbance was recoded at 532 nm using a UV-visible spectrophotometer.

Standard solution of malonaldehyde was prepared by using 1,1,3,3-tetramethoxypropane (TMP). Mixing 164.7 µL of 1,1,3,3- tetramethoxypropane (TMP) in 100 mL of deionized water gave 10 µmol/mL stock solution. Prior to assessment, 200 µL of the stock solution was dissolved in 50 mL of 1% (v/v) sulphuric acid, followed by incubation for 2 hours at room temperature. The prepared malonaldehyde standard was 40 nmol/mL. Subsequently, the 40 nmol/mL malonaldehyde standard was diluted with 1% (v/v) H_2SO_4 to obtain 5, 10, 15, 20 and 30 nmol/mL standards.

3.11. Statistics

Measurements were performed at least thrice on each freshly prepared samples. The results were expressed as mean and standard deviation of three measurements. The significant treatment effects were identified using analysis of variance in SPSS program (v. 21. IBM, USA). Significant ($p < 0.05$) differences between means were detected by means of the Tukey technique (Noshad et al., 2016).

Chapter 4

Results and discussion

The quantity of emulsifier that is required to form a reasonably stable emulsion with anticipated properties is to be known from a practical viewpoint. In order to form a stable tertiary emulsion, it is desired that a reasonably steady primary emulsion droplets with sufficiently large positive charge and small droplet size at pH 3.0 is formed. The purpose of these experiments was to arrive at a suitable sodium caseinate concentration at 1% (w/v) oil concentration based on particle size and ζ-potential. If caseinate concentration is too low, sufficient coating of oil droplet may not be feasible resulting in an emulsion structure that collapses due to bridging flocculation. On the other hand, if caseinate concentration is higher than necessary, non-adsorbed biopolymer will be present in aqueous phase as sub-micelles and may cause depletion flocculation. In such cases, depletion forces may override the stability forces such as repulsive and steric forces. The concentration of sodium caseinate that allowed adequate coating of oil droplets and prevented excess occurrence in aqueous phase was considered appropriate (Dickinson et al., 2003). The Z- Average size and ζ-potential of emulsions made up of a single emulsifier caseinate at concentrations varying from 0.2 to 1.2% (w/v) were measured within 24 hours of emulsion preparation. Figure 4.1 shows the effect of variation of caseinate concentration on the particle size and ζ-potential of primary emulsions.

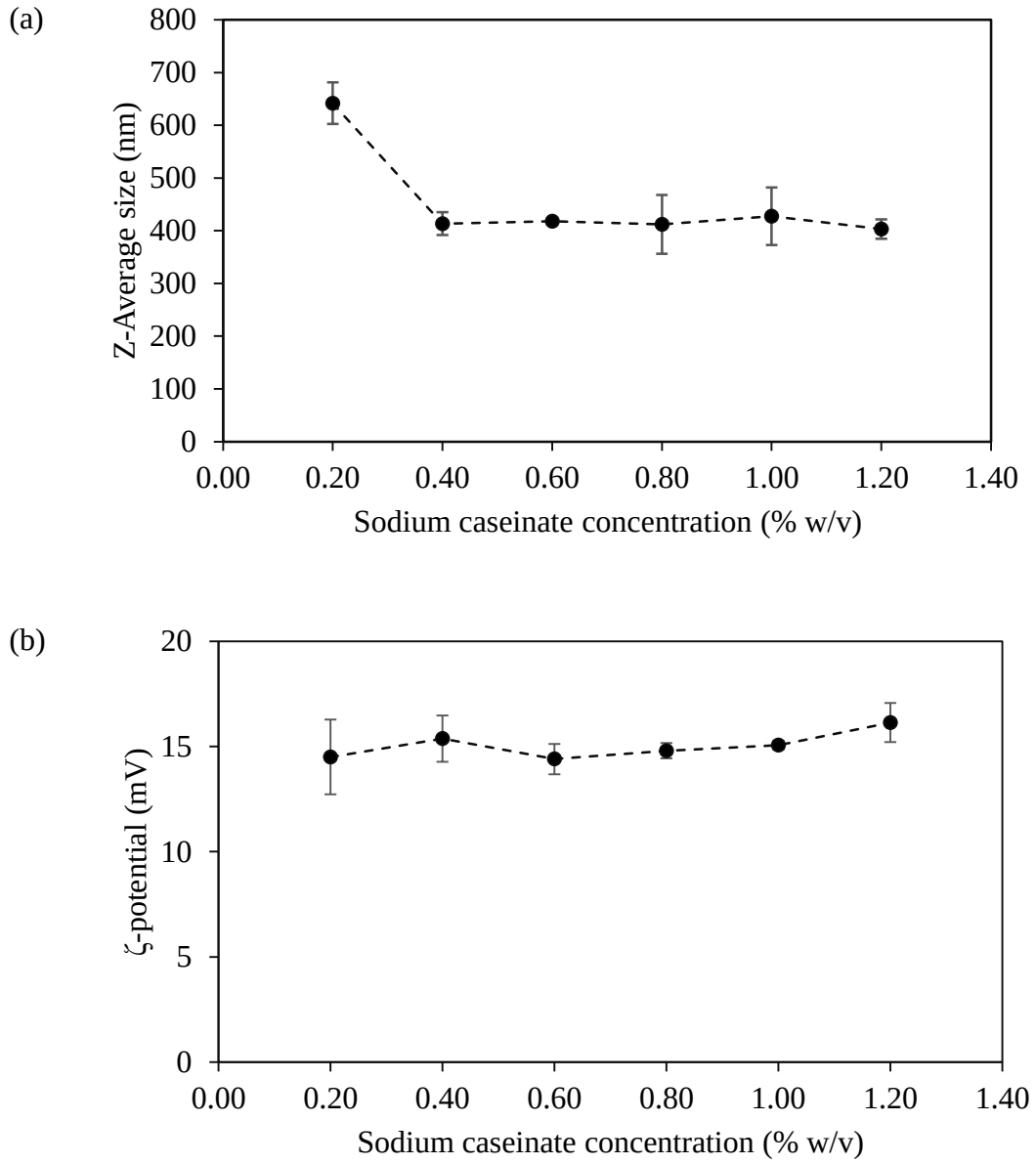


Figure 4.1: Dependence of sodium caseinate concentration on (a) particle size and (b) ζ-potential of primary emulsions (1% flaxseed oil, 50 mM acetic acid buffer, pH 3). Data represents mean ± standard deviation (n=3).

There were significant differences (p<0.05) in particle sizes of primary emulsions due to variation in concentration of emulsifier. Sodium caseinate stabilized flaxseed oil-in-water emulsions had a constant Z-Average size around 400 nm for emulsifier concentrations 0.4, 0.6, 0.8, 0.10 and 0.12% (w/v) as observed from Figure 4.1. This observation was consistent with the outcomes of experiments carried out by Sánchez and Patino (2005) at pH 7.0 and Srinivasan et al. (1999). However, at 0.2% (w/v) caseinate concentration, the emulsion size elevated to 641.7 nm (polydispersity index, PDI<0.4) possibly due to insufficient emulsifier to fully coat the oil surface that led the droplets to

increase the size in order to reduce the surface area that required protein coverage. Therefore, it can be inferred that a critical concentration of emulsifier is required to coat the oil droplets that gives minimum Z-Average size. Further increase in the concentration of caseinate does not yield smaller droplets under the prevailing pH 3.0, 50 mM acetate buffer and emulsion forming conditions but may form multilayers on the surface or remain in solution (Sánchez and Patino, 2005). Therefore, in this study, the concentration of 0.4% (w/v) was chosen as an ideal concentration for primary emulsion formation. This is supported by the observation that the emulsion stabilized with more protein did not yield notably higher positive ζ-potential.

Subsequently, secondary emulsions were prepared by trial and error using varied concentration (0.25% and 0.50%) of sodium alginate. Visible aggregation was a major problem in the formation of multilayer emulsion as illustrated in figure 4.2. This problem was attributed to the high salt concentration of buffer. Studies conducted by Zeeb et al. (2013) supported the present observation that multilayer emulsions destabilized at higher citrate buffer strength. The issue was solved by reducing the concentration of acetate buffer to 5 mM. A second layer concentration of 0.25% (w/v) was sufficient to bring about charge reversal -39.1 ± 0.80 mV indicating the adsorption of alginate on primary droplets.

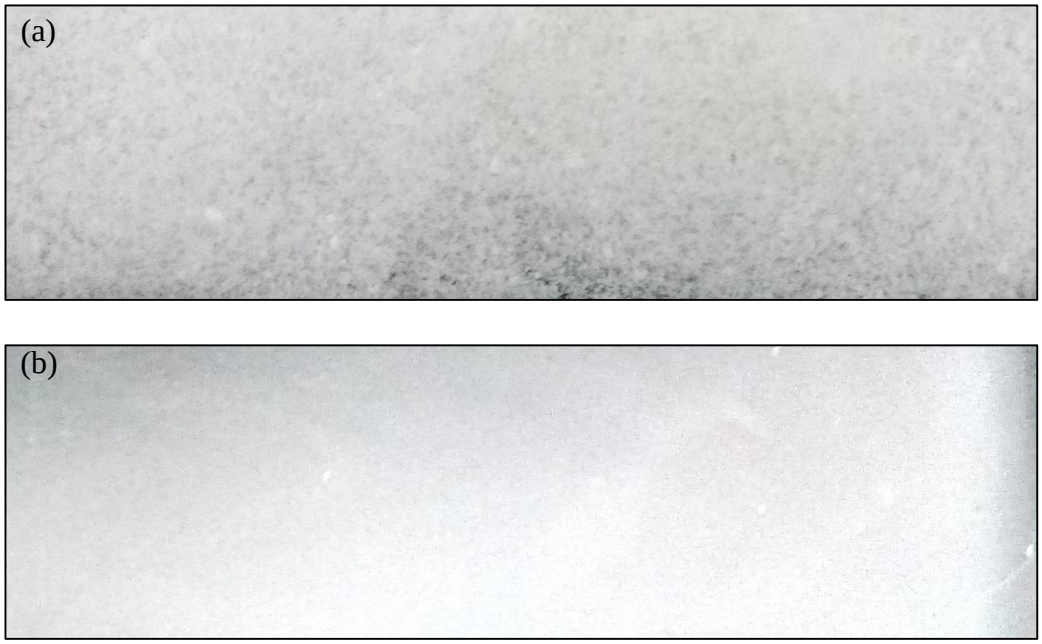


Figure 4.2: Effect of buffer on multilayer emulsion preparation. (a) The formation of aggregates during bilayer emulsion preparation using acetate buffer at 50 mM. (b) Stable bilayer emulsions of sodium caseinate and alginate formed using acetate buffer at 5 mM concentration.

Chitosan of various concentration was added to bilayer emulsions stabilized by sodium caseinate-alginate in order to identify the concentration suitable for the formation of trilayer emulsion (sodium caseinate-alginate-chitosan). The effect of chitosan concentration on droplet characteristics (particle size and ζ-potential) and physical stability are discussed in the following three sub-sections.

4.1. ζ-potential

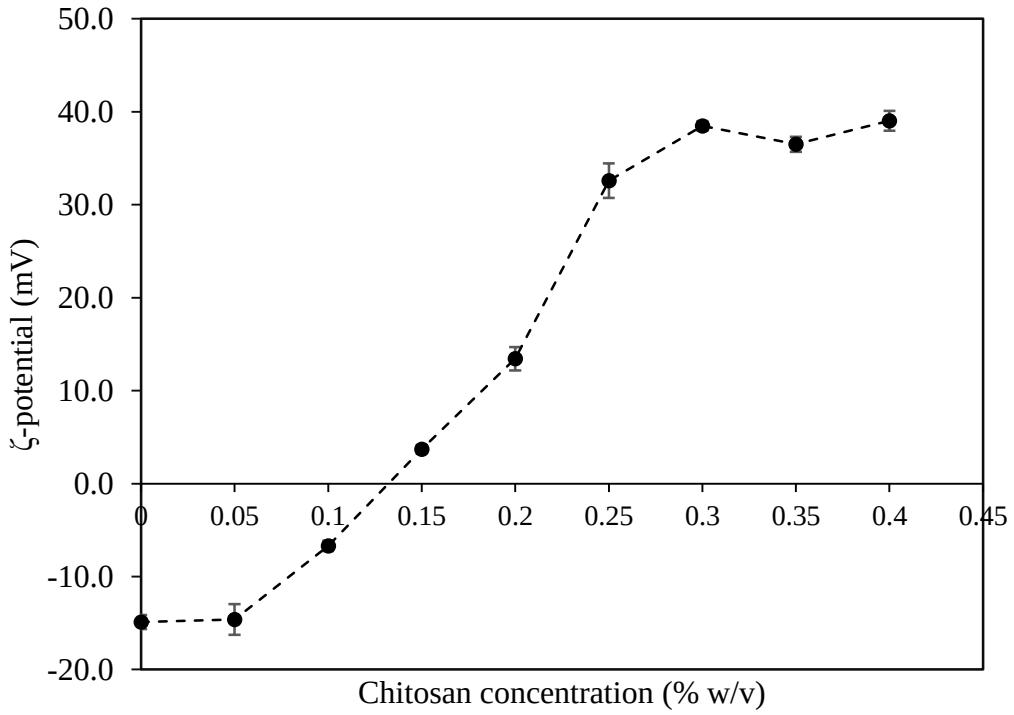


Figure 4.3: Dependence of ζ-potential on chitosan concentration for tertiary emulsions (1 % w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate). Data represents mean ± standard deviation (n=3).

ζ-potential measurements were conducted in order to ascertain the electrostatic deposition of chitosan on interfacial membrane consisting of caseinate- alginate surrounding flaxseed oil droplets (secondary emulsion). The influence of chitosan (CH) on ζ-potential of secondary emulsions stabilized by interfacial materials sodium caseinate (SCN) - sodium alginate (SAG) is shown in figure 4.3. The tertiary emulsion bound by SCN-SAG-CH membrane was prepared with varying concentration of chitosan (0 to 0.4 % w/v). As indicated by figure 4.3, when the chitosan concentration was zero, that is, the protocol for diluting the SCN-SAG with third biopolymer was conducted in the absence of chitosan, ζ-potential was -14.9 ± 0.75 mV. This indicated that SCN-SAG interfacial film had a negative charge. It could be explained by the fact that alginate being the second

layer, possessed a net negative charge at the pH studied and therefore, secondary emulsions in the absence of chitosan formed negatively charged oil droplets. With increasing addition of chitosan (0.05 to 0.25% w/v), ζ-potential of flaxseed oil droplets became progressively less negative. Eventually, the droplet charge reversed from negative to positive. It is supposed that the positively charged biopolymer chitosan adsorbed onto the oil surface coated with SCN-SAG having overall negative charge. This resulted in the formation of tertiary emulsions with SCN-SAG-CH interface. When the chitosan concentration was around 0.13 % (w/v), there was no charge on the flaxseed oil droplets suggesting that adequate quantity of chitosan had adsorbed to counteract the charge on the initial oil globule. ζ- potential became constant around +33 mV when chitosan concentration surpassed 0.25 % (w/v), indicating that the flaxseed oil droplets were saturated with third layer material, chitosan.

4.2. Droplet size

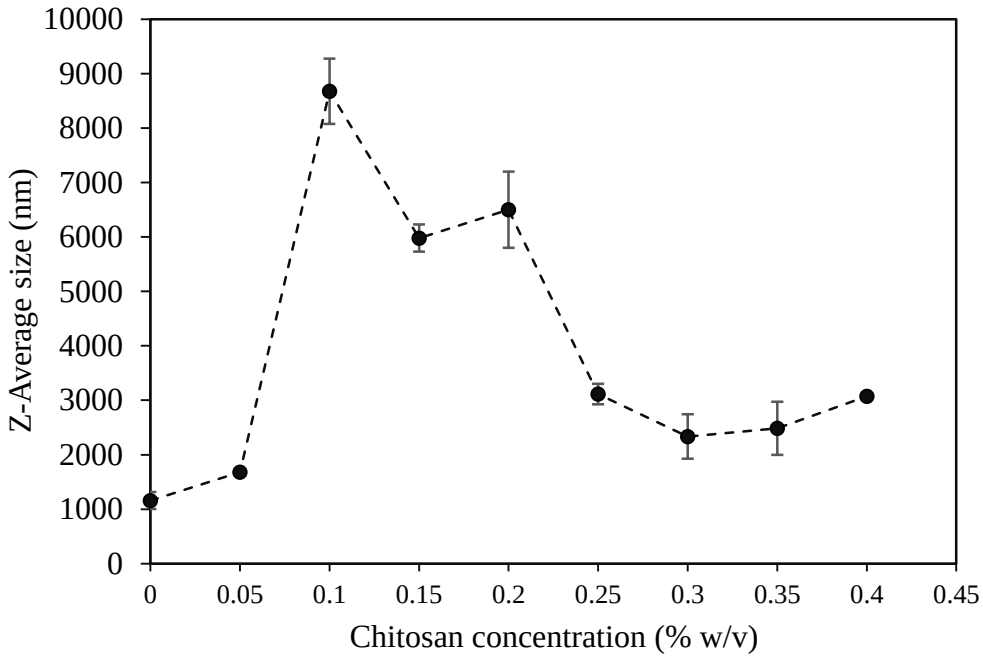


Figure 4.4: Dependence of particle size (Z-Average) on chitosan concentration for tertiary emulsions (1 % w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate). Data represents mean ± standard deviation (n=3).

Figure 4.4 shows the dependence of droplet size of SCN-SAG stabilized flaxseed oil emulsions on the chitosan concentration. Without chitosan, the Z-Average size was very small at 1159 ± 154.26 nm and there was no aggregation. However, the presence of chitosan increased the mean droplet diameter significantly ($p< 0.05$). At low chitosan concentration (0.05 to 0.20% w/v), there were significant growth in the size of droplets. It

could be explained by the fact that the added chitosan interacted with the charged surface of SCN-SAG stabilized flaxseed oil droplets, thus inducing destabilization because of neutralization of charge. The induced charge neutralization makes the droplets to come nearer, encouraging the development of droplet aggregates with bigger sizes. In addition, the droplet aggregation could have occurred because of inadequate chitosan present to completely coat the SCN-SAG stabilized flaxseed oil droplets. In this situation, it is probable that chitosan could act as a bridge connecting with more than one alginate molecule present on surface of two or more different droplets, thus bringing them closer. Therefore, the droplet size increase could be attributed to the attractive electrostatic forces induced by the phenomenon similar to bridging flocculation among flaxseed oil droplets. Ogawa et al. (2004) proposed that droplet collision occurred quickly while adsorption of third biopolymer on droplet until saturation occurred slowly. The consequential time lapse caused bridging flocculation.

With the further increase in chitosan concentration (0.25 to 0.4% w/v), the droplet size was fairly constant. It could be proposed that addition of chitosan above 0.25% (w/v) had adequately coated the flaxseed oil droplets stabilized by negatively charged SCN-SAG membrane and thus formed a thick trilayer interfacial film on flaxseed oil droplets. Similar behavior could be observed by the interaction of bilayer membranes of lecithin- chitosan deposited with a third material pectin (Ogawa et al., 2004).

4.3. Physical stability of emulsions

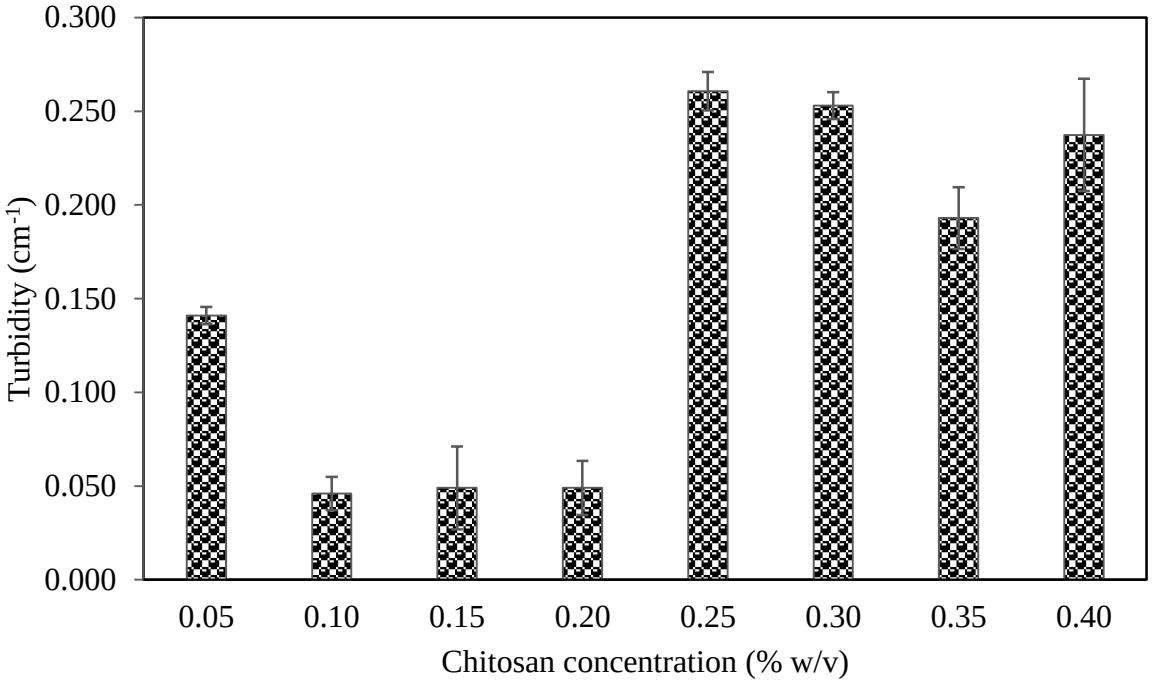


Figure 4.5: Dependence of emulsion physical stability (creaming and sedimentation) on chitosan concentration for tertiary emulsions (1% w/v flaxseed oil, 0.4% w/v sodium caseinate, 0.25% w/v sodium alginate). The stability to phase separation was determined as turbidity at 600 nm measured at about 30% of emulsion height after undisturbed storage for 24 hours. Lower turbidity indicated unstable emulsions. Data represents mean ± standard deviation (n=3).

The turbidity of tertiary emulsions containing different chitosan concentration (0.05 to 0.40 % w/v) were recorded 1 day after preparation of diluted emulsions. Statistically, significant turbidity differences (p<0.05) were obtained for tertiary emulsions with varying chitosan concentration. From figure 4.5, the turbidity of tertiary emulsions with chitosan concentration 0.10%, 0.15% and 0.20 % had significantly lower turbidity. This result was obtained because a clear droplet depleted region was formed at 30% emulsion height, where the light passed due to oil aggregate sedimentation. This is the region where charge neutralization between oil droplets and chitosan occurred (section 4.1). All other emulsions were stable to creaming and sedimentation as observed as higher turbidity. In addition to turbidity, creaming stability studies showed no visible separation of cream and serum layers after 7 days of storage.

From the above discussion, 0.25% chitosan was considered appropriate for the formation of tertiary emulsions. In the remaining study, tertiary flaxseed oil emulsions were prepared using 0.25 % (w/v) chitosan as third layer material because stable emulsions with droplets having high ζ-potential and relatively small droplet size were

obtained. SCN, SCN-SAG and SCN-SAG-CH were designed as interfaces for primary, secondary and tertiary emulsion enclosed by one, two and three layer membrane, respectively. Further, comparison between primary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate), secondary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate) and tertiary emulsions (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate + 0.25% w/v chitosan) were carried out based on their response to stresses and oxidative stability.

The initial properties of primary, secondary and tertiary emulsions are illustrated in figure 4.6. The pH of the final primary, secondary and tertiary emulsions were 3.55, 3.76 and 3.47 respectively. The particle sizes of primary, secondary and tertiary emulsions show large variation with the primary emulsions having the smallest and tertiary emulsion having the largest size. This is due to fact that sodium caseinate, being a protein had formed small sized droplets of 295.17 ± 1.8 at pH 3. Proteins have smaller molecular sizes than polysaccharides. On the other hand, secondary emulsions have an additional layer of sodium alginate over caseinate on the interface thus increasing the size of oil droplet to 893.33 ± 41. Further, chitosan addition to form tertiary emulsions increased the size to 3114 ± 187 nm.

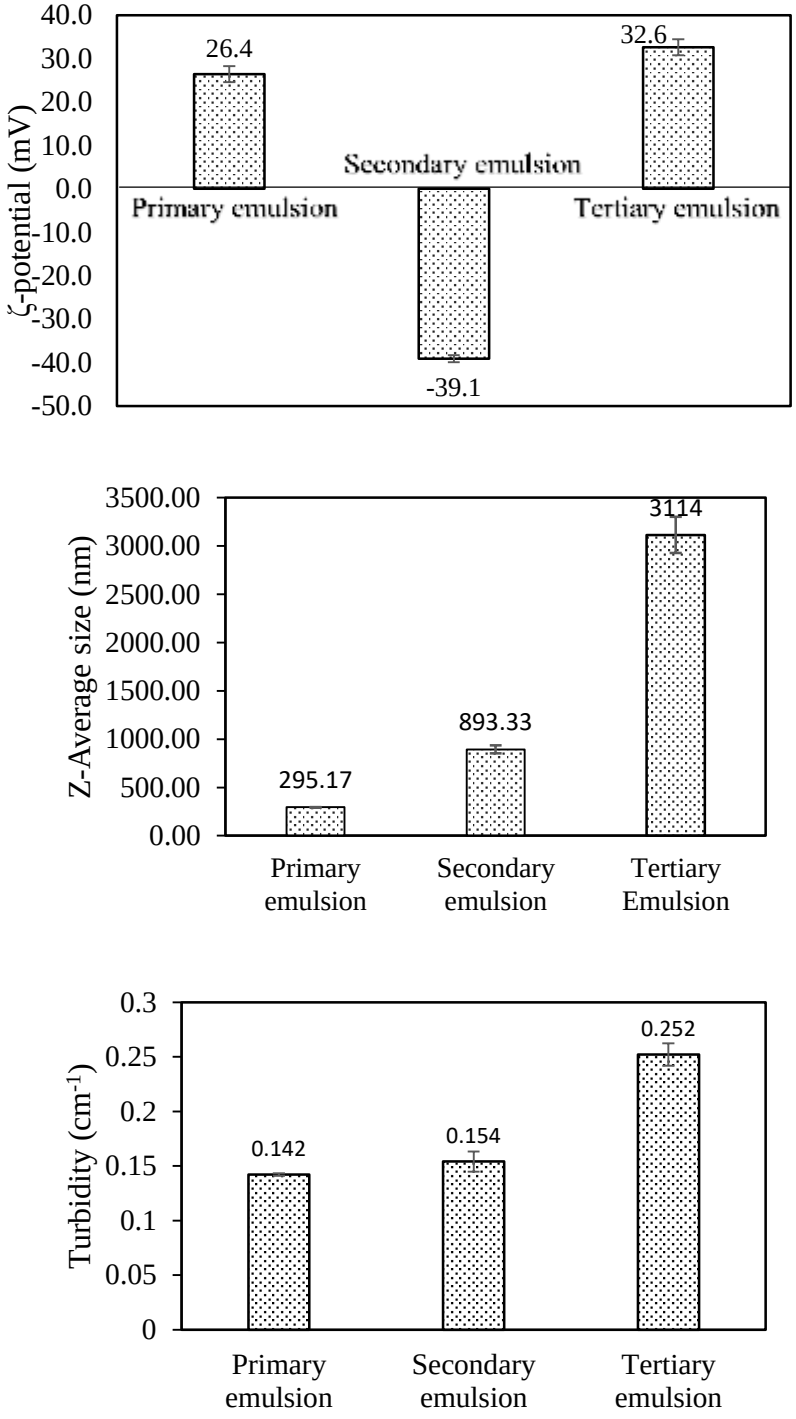
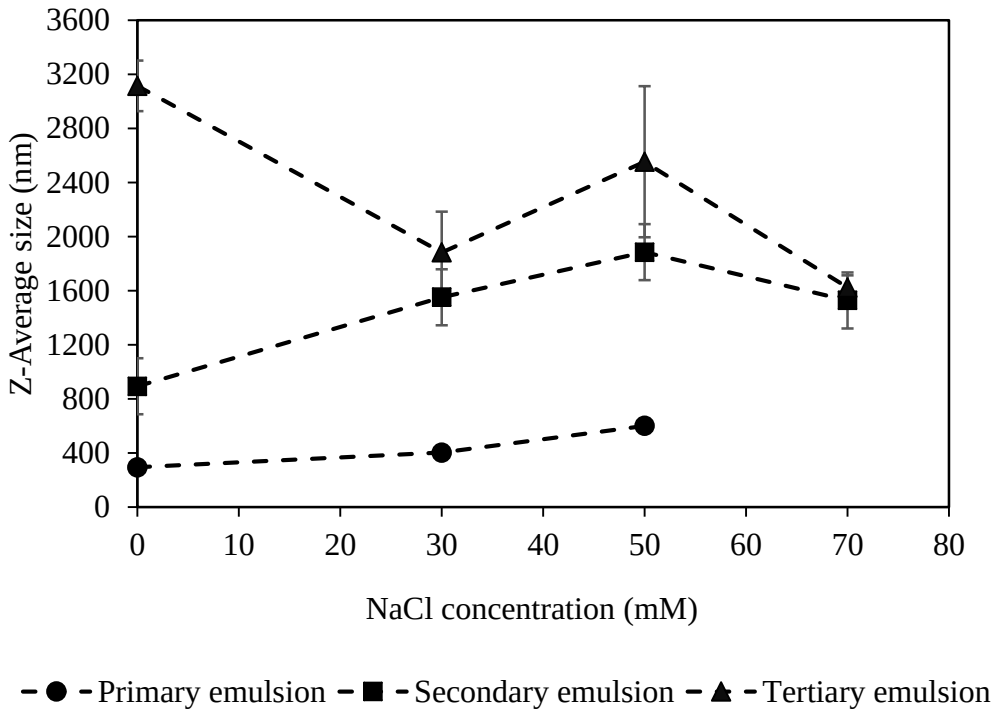


Figure 4.6. (a) Comparison of ζ-potential of primary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate), secondary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate) and tertiary emulsions (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate + 0.25% w/v chitosan). (b) Comparison of Z-Average sizes of primary, secondary and tertiary emulsions. (c) Comparison of turbidity of primary, secondary and tertiary emulsions. Data represents mean ± standard deviation (n=3).

4.4. Effect of salt

The influence of addition of sodium chloride (30 to 70 mM) on the particle size and ζ-potential of emulsions was studied as indicated in figure 4. 7. Presence of salt changes the ionic strength of aqueous phase that in turn affect emulsion stability. The motive of conducting these experiments was to determine the effect ionic strength has on the structural integrity of one, two and three layer coated emulsion droplets. It must be pointed out that the buffer components will also add up to the total ionic strength of the aqueous phase. Nonetheless, the ionic strength must be predominantly determined by the added salt, specifically at higher concentration (Güzey and McClements, 2006).

There were significant differences ($p<0.05$) in particle sizes of primary, secondary and tertiary emulsions due to addition of sodium chloride. The secondary and tertiary emulsions were relatively resistant to droplet aggregation in all the experimented salt concentrations (30, 50 and 70 mM). Though there were fluctuations in Z- Average size due to slight aggregation, comparatively stable secondary and tertiary emulsions were obtained as indicated by figure 4.7 (a). The plausible explanation for this observation is that secondary and tertiary interfacial layers were thick enough to provide satisfactory steric stabilization. Conversely, the primary emulsion of sodium caseinate was stable only at salt concentration less than 50 mM. However, the primary oil droplets remained fairly stable, unaffected by salt addition till 50 mM. Similar trend was exhibited by the results of Srinivasan et al. (2000) where addition of sodium chloride up to 1000 mM concentration to 30% soybean oil-in-water emulsions stabilized by 1% or 3% sodium caseinate at pH 7.0 produced droplets with comparable sizes. Likewise, caseinate stabilized 20% soya oil-in-water emulsions at pH 7.0 produced similar sized droplets with the addition of potassium chloride (KCl) salt up to 200 mM concentration. Conversely, at 70 mM salt addition to primary caseinate emulsion, the droplet coagulation was so extensive that it was not possible to make reliable particle size measurements using diffraction technique. This difficulty might have occurred either due to extensively large size of coagulates or low concentration of particles that a scattering pattern distinct from background could not be acquired. In fact, the coagulum could be viewed with unassisted eye indicating that the particles were above 100 µm size (Ogawa et al., 2004).



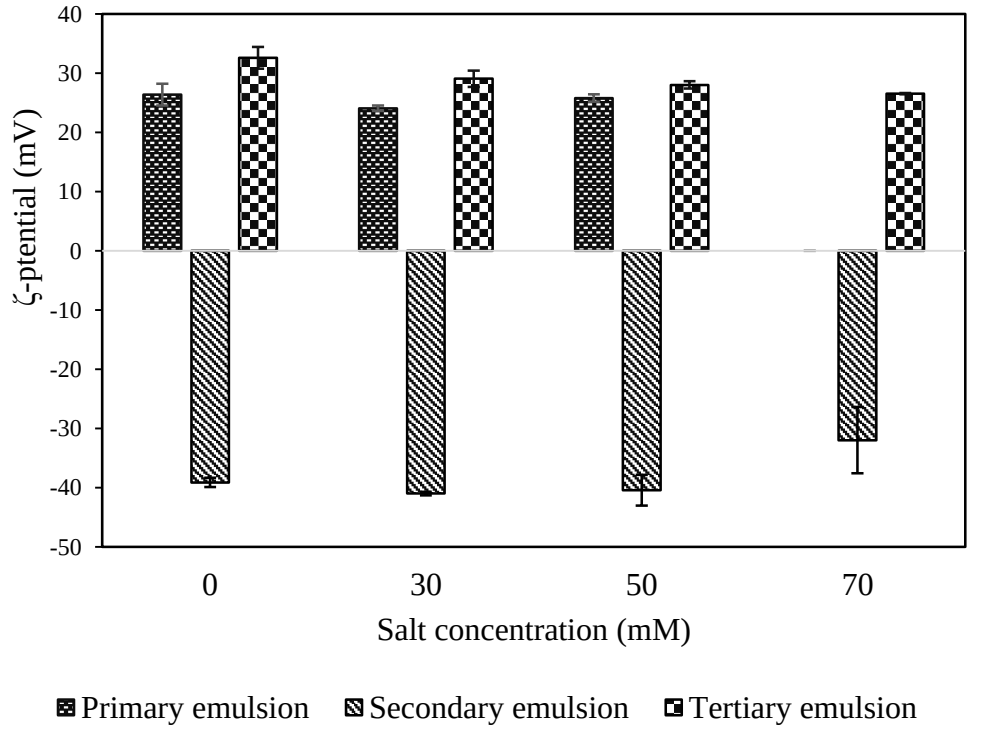


Figure 4.7: (a) Effect of added salt on Z-Average size of primary, secondary and tertiary emulsions. (b) Effect of added salt on ζ-potential of primary, secondary and tertiary emulsions. Data represents mean ± standard deviation (n=3).

The addition of salt (0, 30, 50 and 70 mM) to secondary and tertiary emulsions affected their ζ-potential significantly ($p<0.05$). There was no significant difference by salt addition (0, 30 and 50 mM) on ζ-potential of primary emulsion. The ζ-potential of the primary sodium caseinate stabilized emulsions were positive at all salt concentrations (0, 30 and 50 mM). In addition, the magnitude of ζ-potential remained fairly constant, unaffected by salt addition till 50 mM. However, primary emulsions were physically unstable and extensively coagulated at 70 mM NaCl, rendering them unavailable to sampling and ζ-potential measurements. This could be explained by the behavior of proteins constituting sodium caseinate. It has been observed that a compositional balance is required for the stability of sodium caseinate emulsified colloidal systems. Casanova and Dickinson (1998) has observed that emulsions stabilized completely by αs_1-casein had poor salt stability. However, salt stability greatly improved when one-third of αs_1-casein was substituted with β-casein fraction. Increasing ionic strength increases adsorption of αs_1-casein relative to β-casein on oil droplet surfaces (Dickinson, 2010). Therefore, it can be assumed that this compositional balance required in caseinate stabilized emulsions was disrupted when salt above 70 mM was added to primary sodium caseinate emulsions.

The ζ-potential of sodium caseinate-sodium alginate stabilized droplets in the secondary emulsions exhibited negative potential when salt of various concentration was added. The magnitude of the ζ-potential remained relatively constant up to a concentration of 50 mM and dropped remarkably for 70 mM sodium chloride addition. The observation that ζ-potential of secondary emulsions remained notably unaltered when it would be expected to be decreasing due to salt induced screening effects is attributed to the change in interface thickness. On further addition of salt, the ionic strength increased making the interaction between anionic alginate and cationic caseinate to weaken due to electrostatic screening effect thus making the interface less compact. At elevated salt concentration of 70 mM resulting in enhanced ionic strength, desorption of alginate from the surface of secondary emulsions caused the droplets to lose its negative charge. Alginate desorption would have occurred due to weakened electrostatic interactions in the presence of interfering salt (Güzey and McClements, 2006).

The ζ-potential of sodium caseinate-sodium alginate-chitosan stabilized oil droplets of tertiary emulsion exhibited positive ζ-potential when salt of various concentration was added. The magnitude of ζ-potential slightly decreased in magnitude with increasing concentration of sodium chloride as shown in figure 4.7 (b). These results

were consistent to the trend obtained by Güzey and McClements (2006) for β-lactoglobulin-pectin-chitosan stabilized tertiary emulsion droplets of corn oil. Even at the highest concentration of added sodium chloride, the ζ-potential was relatively large at 26.57±0.12 mV. However, this emulsions was not completely free from droplet aggregation. The observation that ζ-potential had only a minor effect by salt addition could suggest that electric potential was less influenced by electrostatic screening. The inconspicuous effect of electrostatic screening in tertiary oil droplets could be partly contributed by the thick interfacial membrane constituted by the three layers caseinate-alginate-chitosan. The ζ-potential measurements made on a relatively thick interfacial membrane consisting of several interacting biopolymers must be considered with caution as the plane of shear might not be well defined. Plane of shear refers to the region where counter ions are held firm onto the droplet surface as the droplet moves around the continuous water phase (Güzey and McClements, 2006).

4.5. Effect of temperature

The motive of conducting these experiments was to determine the effect of temperature variation on the structural integrity of primary, secondary and tertiary emulsions. Temperature fluctuations as encountered during thermal processing of food affect the stability and integrity of emulsions. Primary (sodium caseinate), secondary (sodium caseinate-sodium alginate) and tertiary (sodium caseinate- sodium alginate-chitosan) emulsions of same oil concentration 1% (w/v) were held at temperatures ranging from 30 to 90 °C for an interval of 30 minutes and cooled immediately. The influence of temperature variation on the particle size and ζ-potential of emulsions was examined as indicated in figure 4.8. In addition, phase contrast microscopic images of temperature treated emulsions are indicated in figure 4.9.

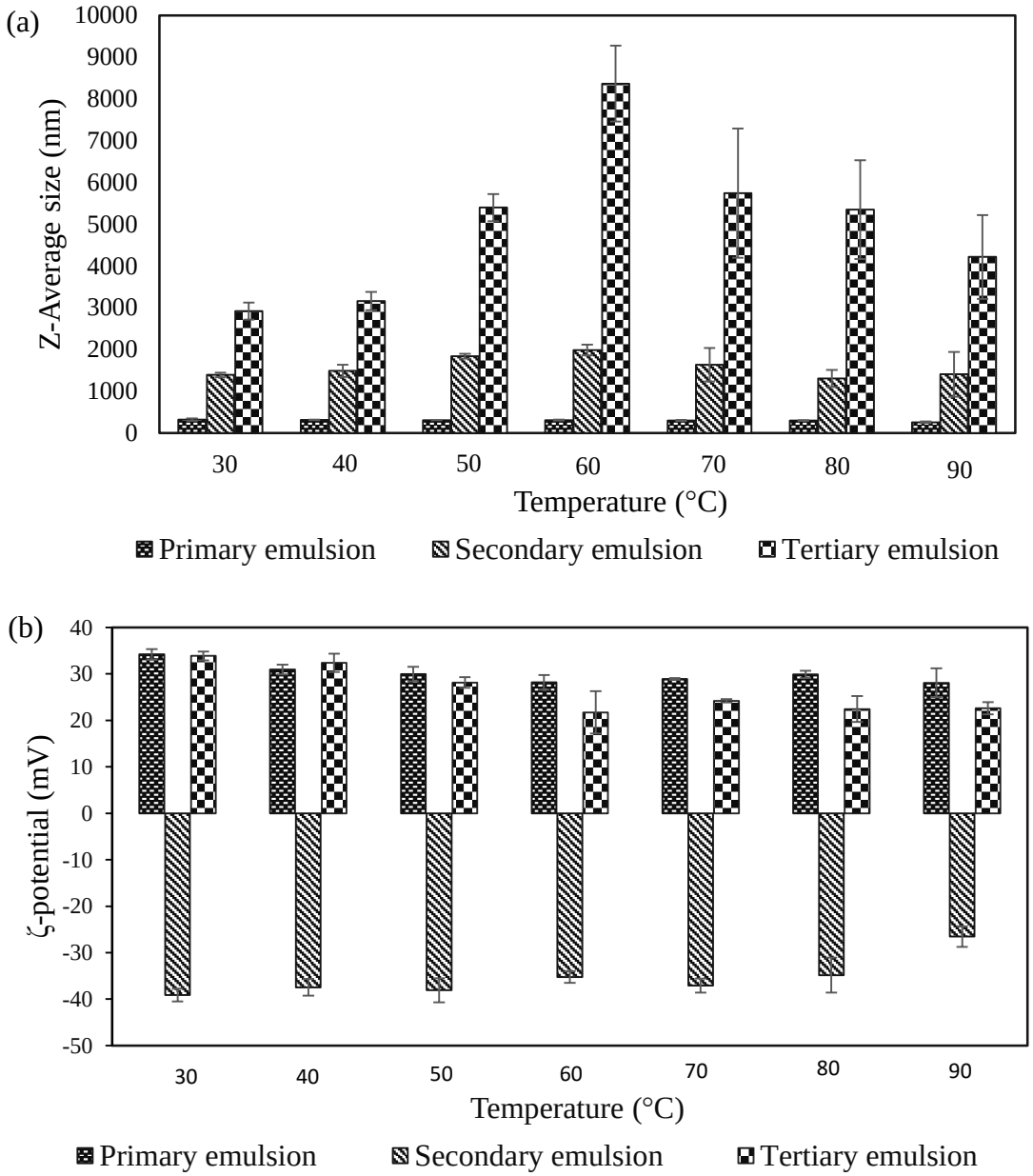


Figure 4.8: (a) Effect of temperature on Z-Average size of primary, secondary and tertiary emulsions. (b) Effect of temperature on ζ-potential of primary, secondary and tertiary emulsions. Data represents mean ± standard deviation (n=3).

There were significant differences ($p<0.05$) in mean particle sizes of primary and tertiary emulsions due to variation in temperature. However, secondary emulsions subjected to temperature variation did not show significant differences in particle sizes. The primary emulsions of sodium caseinate at 5 mM buffer concentration had relatively identical Z-Average sizes for all evaluated temperatures (Figure 4.8 (a) and Figure 4.9 (a), (b), (c) and (d)). This observation is in contradiction with most protein stabilized interfaces as primary emulsions would be expected to aggregate by bridging flocculation. Generally, in protein stabilized primary emulsions physical stability is hampered by heat induced

dissociation of protein subunits causing bridging flocculation (Noshad et al., 2016). In addition, proximity of the pH to isoelectric point could aggravate droplet aggregation (Güzey and McClements, 2006). It must be noticed that (Güzey and McClements, 2006) used 100 mM sodium acetate buffer at pH 4.0 as aqueous phase. On the other hand, Gu et al. (2005) noticed no significant effect of temperature variation on primary emulsions stabilized by β-lactoglobulin (pH 6, 5 mM phosphate buffer) which is similar to our observation. Therefore, it could be inferred that primary emulsions stabilized by sodium caseinate was stable against droplet aggregation by the fact that droplet repulsive forces were influenced more by ionic strength of aqueous phase rather than temperature fluctuations. Thermal treatment of primary emulsions did not hamper the repulsive forces that kept the oil droplets dispersed throughout the continuous phase.

Secondary emulsions were resistant to temperature change (30 to 90 °C) as no aggregation or rise in particle sizes were recorded. Yang et al. (2015) noted that combining polysaccharide either in conjugated or unconjugated form with protein-stabilized interfaces improved their stability to thermal treatments. Tertiary emulsions resisted aggregation up to 40 °C. On further rise in temperature for the stipulated holding time, the oil droplets bound by trilayer membranes aggregated which was seen as rise in particle sizes with a wide spread and optical images (Figure 4.8 (a) and Figure 4.9 (i), (j), (k), (l)). ζ-potential values of tertiary emulsions suggest that sodium alginate-chitosan complex remained attached to the sodium caseinate coated oil droplets even after heat treatment. The possible explanation for the extensive aggregation of tertiary emulsions is that alginate-chitosan molecules could have undergone certain rearrangements that could have flocculated the oil droplets. For instance, a process analogous to bridging flocculation could have occurred where alginate-chitosan complex could have partially separated from original droplets and could have been shared among two or more droplets. This rearrangement could have been driven by heat induced structural changes in the protein molecules coated over bare oil droplets or variation in hydration and hydrophobicity of chitosan molecules (Güzey and McClements, 2006). Further investigation is necessary to propose a detailed explanation for this phenomenon.

Statistically significant differences ($p<0.05$) in ζ-potential of primary, secondary and tertiary emulsions due to variation in temperature were obtained. ζ-potential of primary emulsion dropped from 34.2 ± 1.13 mV at 30 °C to 28.07 ± 3.11 mV at 90 °C probably due to denaturation of the protein membrane sodium caseinate (Noshad et al.,

2016). However, this minor variation of electric charge on the oil/water interface did not influence the repulsive forces between emulsified oil droplets. The secondary emulsions also maintained the physical integrity as it can be seen that the ζ-potential was similar for temperature rise up to 80 °C. Güzey and McClements (2006) obtained similar results for secondary emulsions composed of β-lactoglobulin-pectin layers. Therefore, in this temperature range, charge on secondary droplets were unaffected by the stress factor. On the other hand, ζ-potential of tertiary emulsions dropped significantly from 33.87 ± 0.96 mV probably due to variation of hydrophilicity and hydration of chitosan with temperature elevation (Noshad et al., 2016).

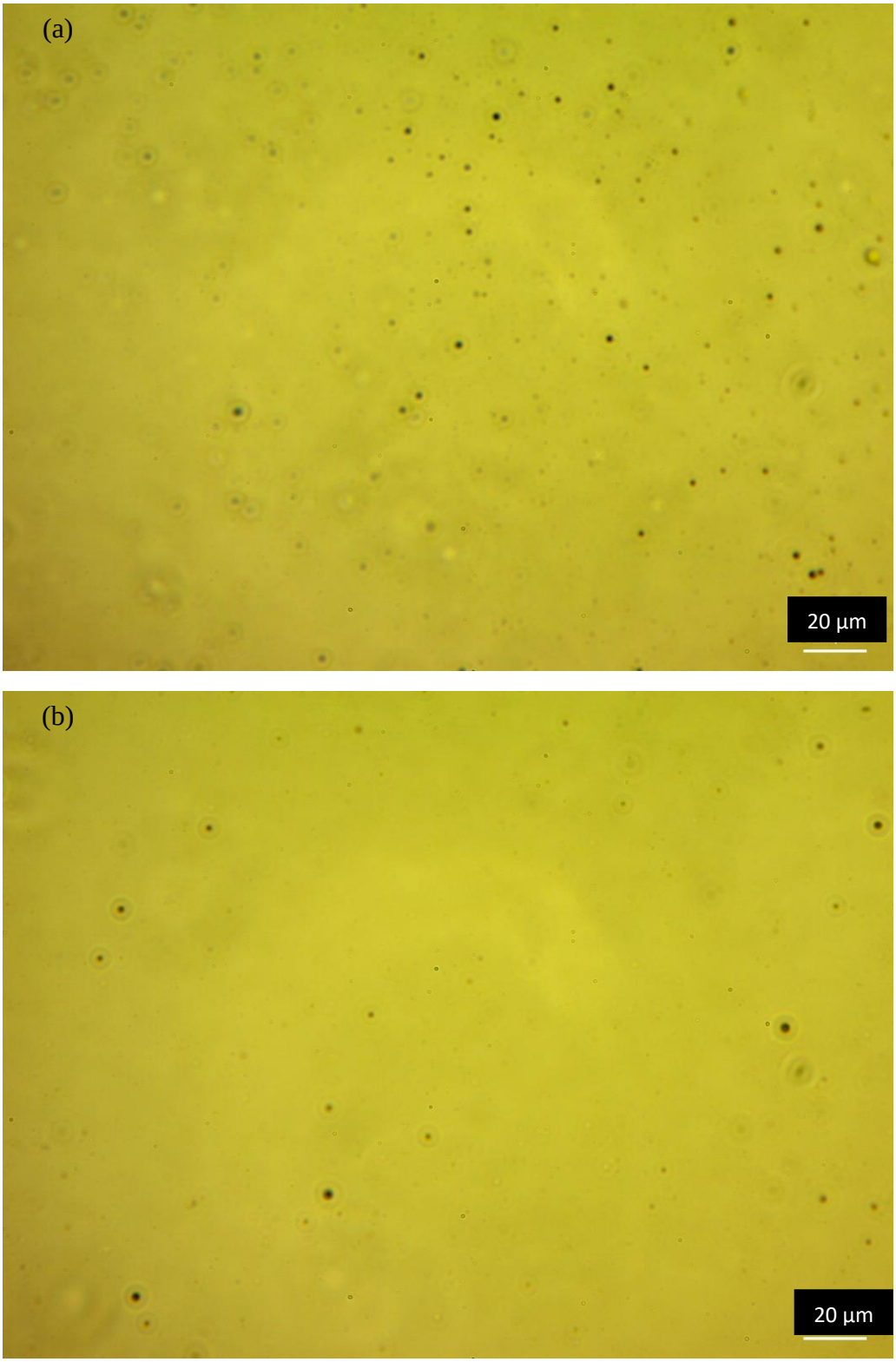


Figure 4.9: Phase-contrast microscopic images of emulsions subjected to temperature variations. (a) Primary emulsion held at 30 °C (b) Primary emulsion held at 50 °C

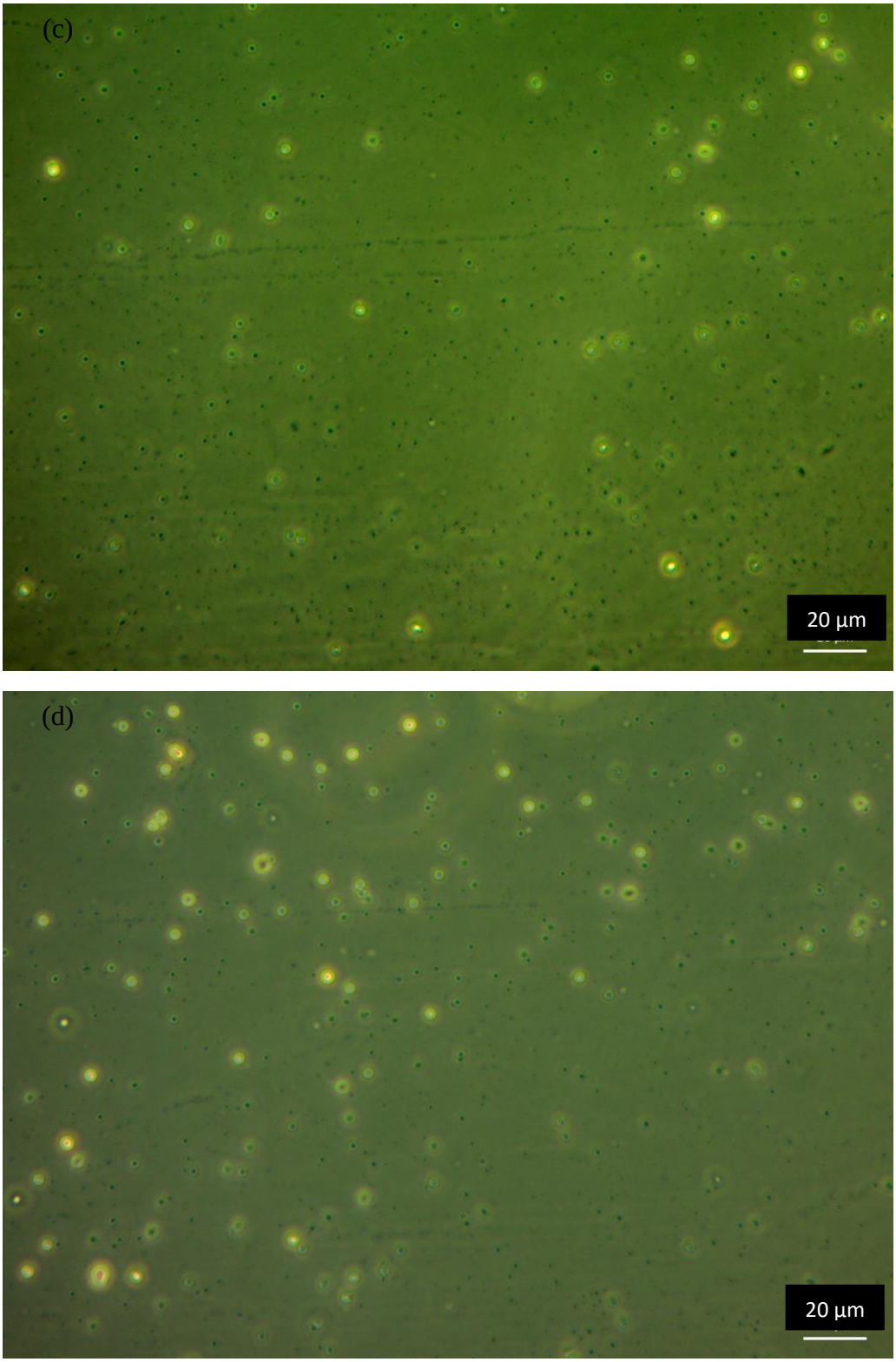


Figure 4.9: Phase-contrast microscopic images of emulsions subjected to temperature variations. (c) Primary emulsion held at 70 °C (d) Primary emulsion held at 90 °C.

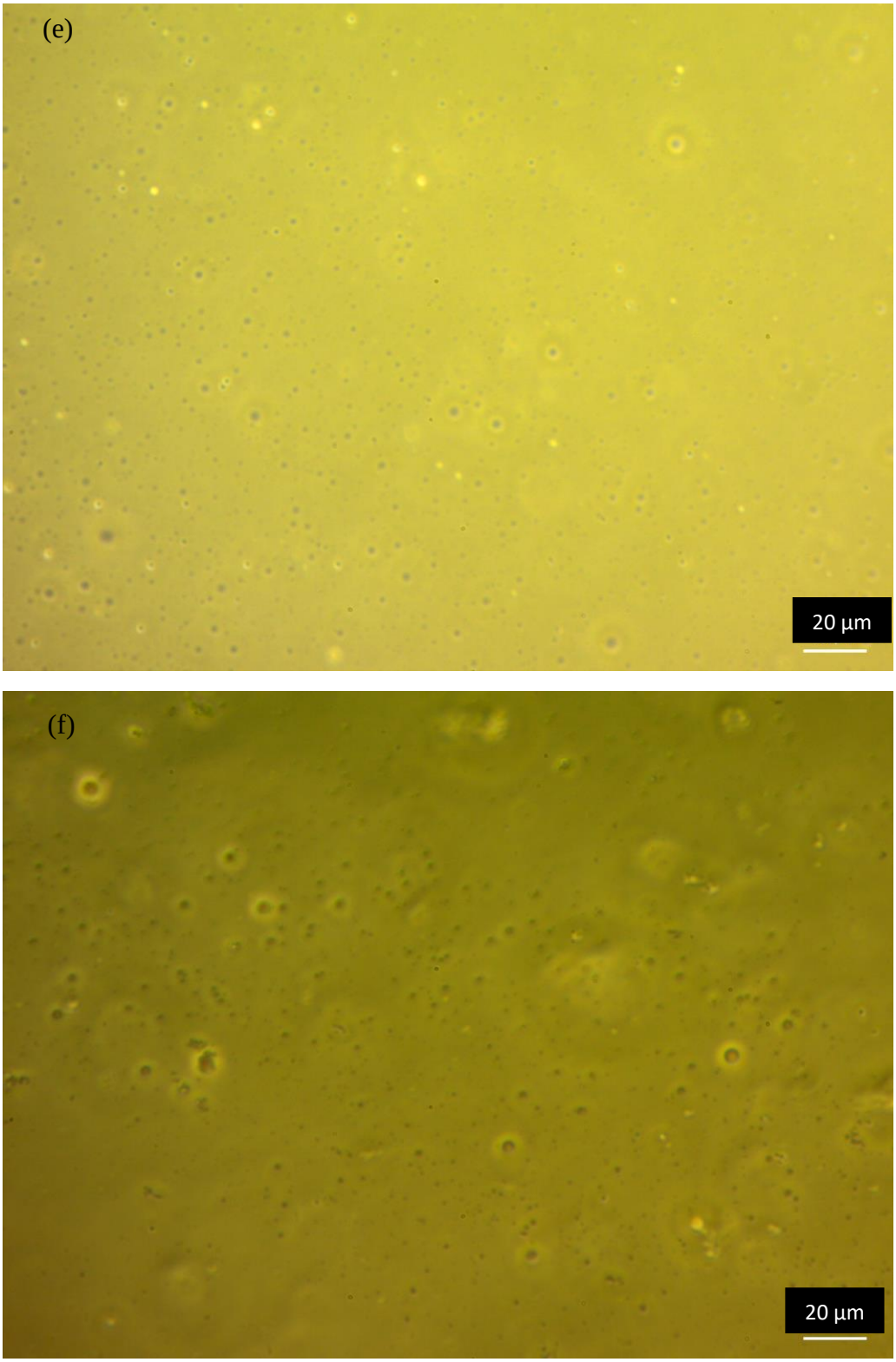


Figure 4.9: Phase-contrast microscopic images of emulsions subjected to temperature variations. (e) Secondary emulsion held at 30 °C (f) Secondary emulsion held at 50 °C.

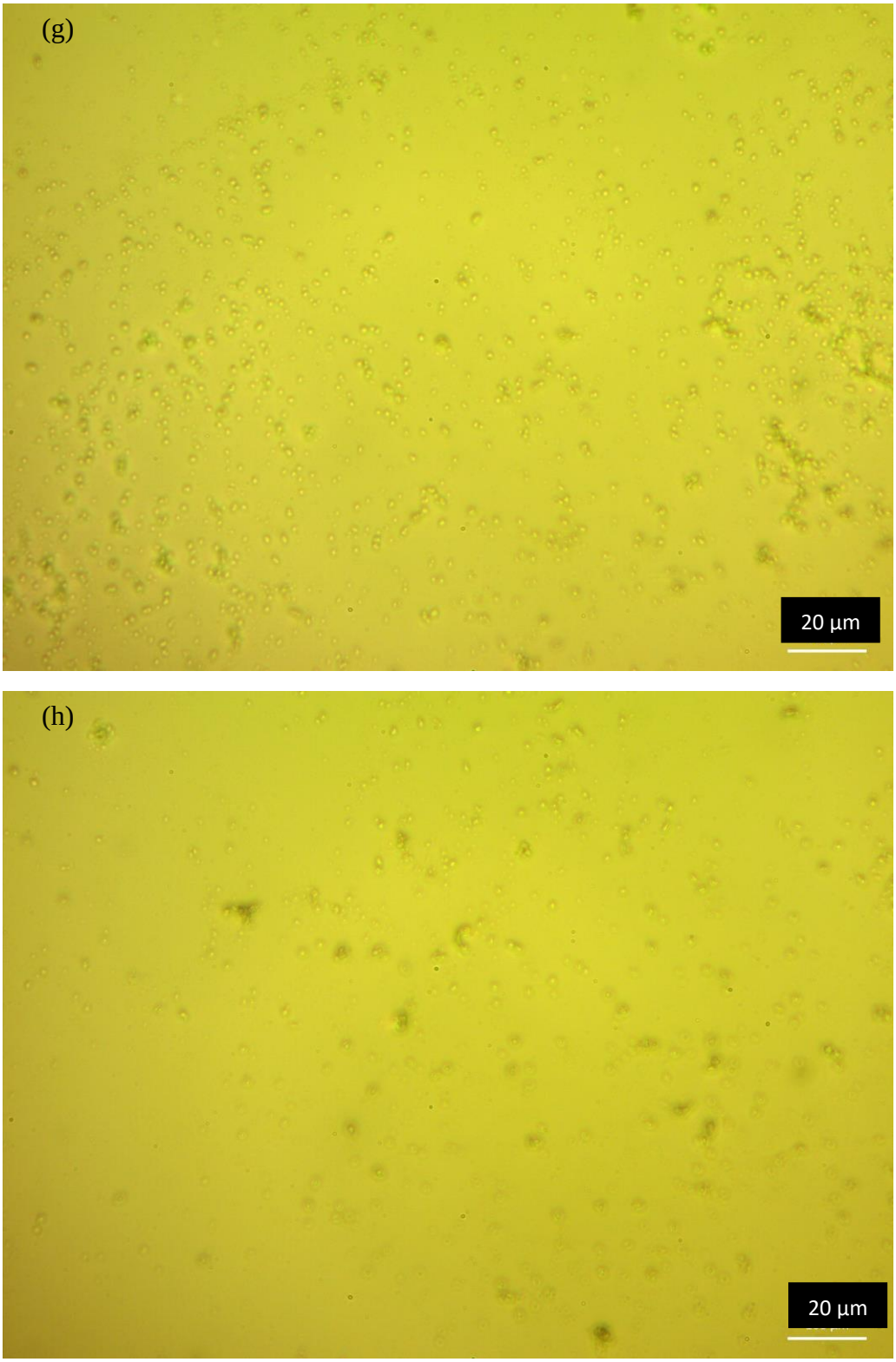


Figure 4.9: Phase-contrast microscopic images of emulsions subjected to temperature variations. (g) Secondary emulsion held at 70 °C (h) Secondary emulsion held at 90 °C.

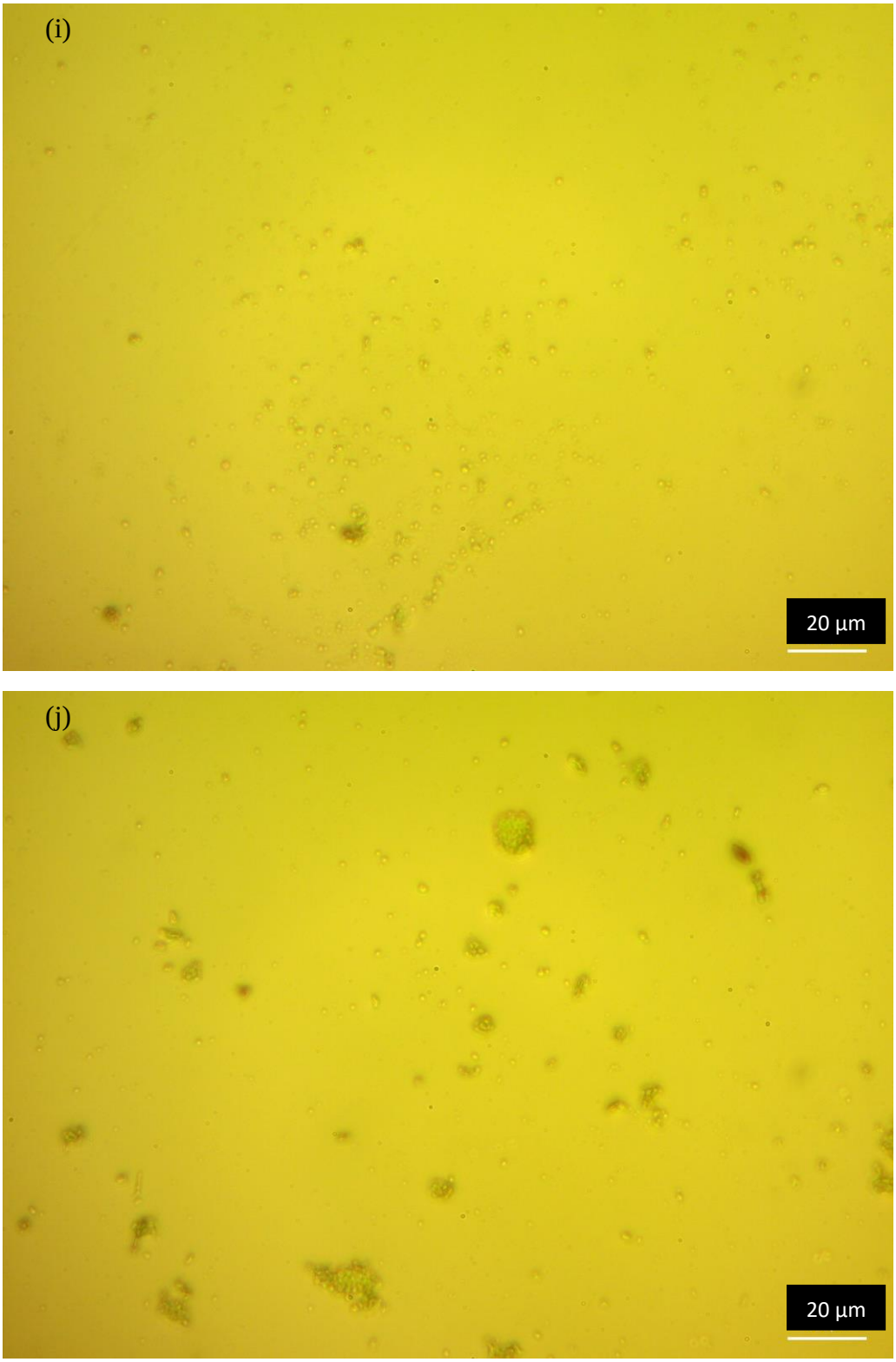


Figure 4.9: Phase-contrast microscopic images of emulsions subjected to temperature variations. (i) Tertiary emulsion held at 30 °C (j) Tertiary emulsion held at 50 °C.

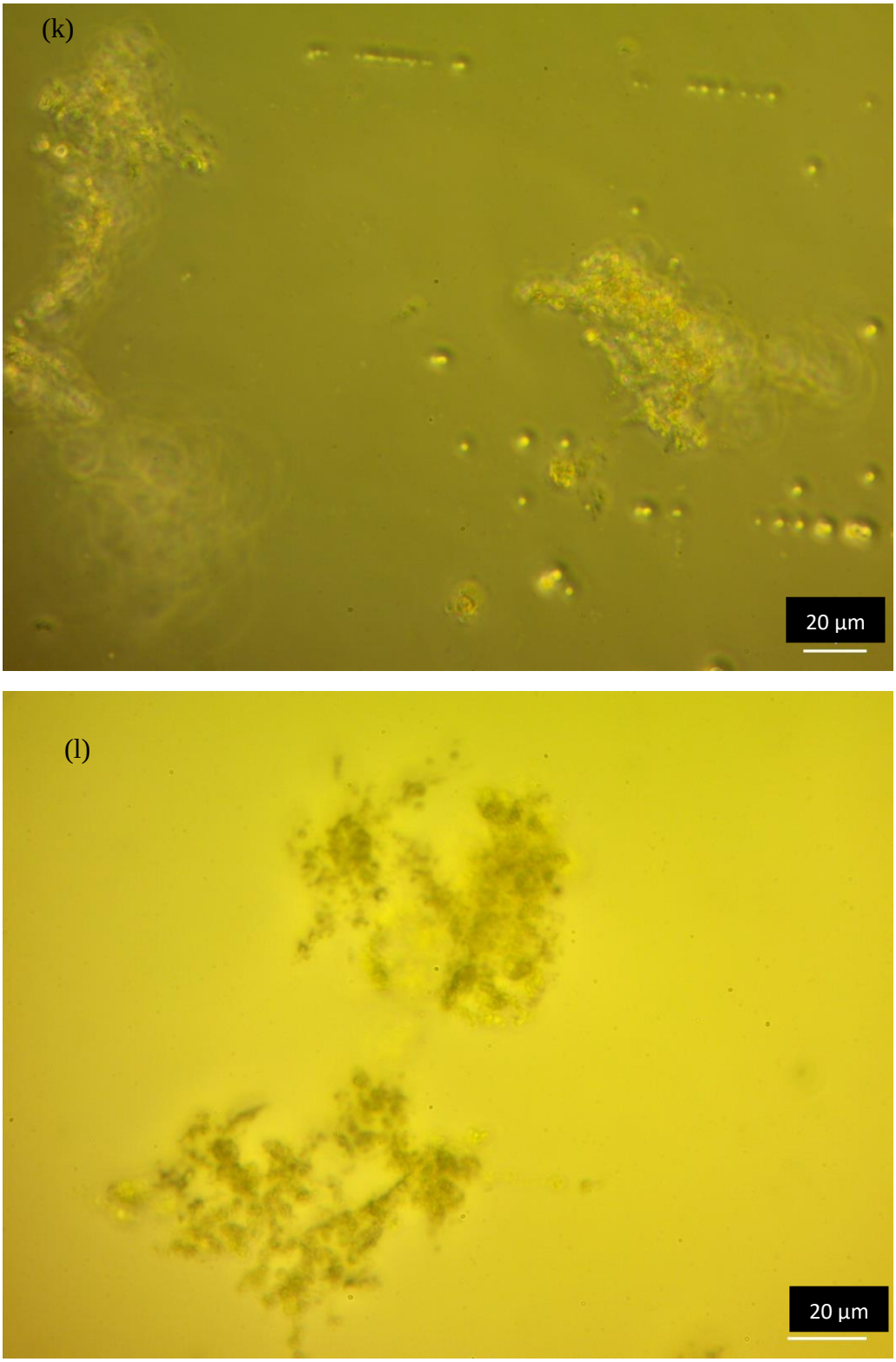


Figure 4.9: Phase-contrast microscopic images of emulsions subjected to temperature variations. (k) Tertiary emulsion held at 70 °C (l) Tertiary emulsion held at 90 °C.

4.6. Oxidative stability

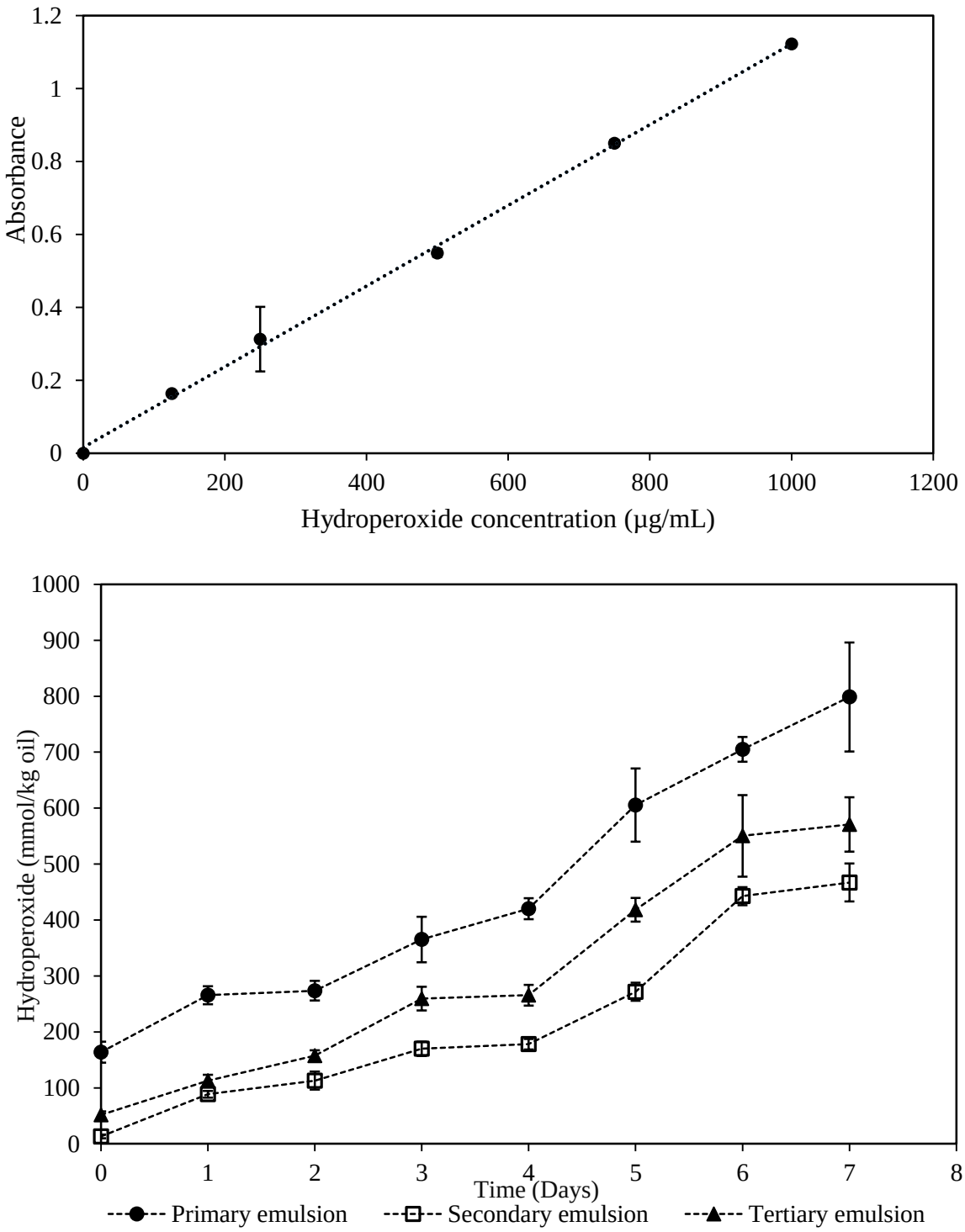


Figure 4.10: (a) Calibration curve used for the determination of hydroperoxide concentration. (b) Comparison of lipid hydroperoxide concentration detected in primary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate), secondary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate) and tertiary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate + 0.25% w/v chitosan) emulsions at 30 °C. Data represents mean ± standard deviation (n=3).

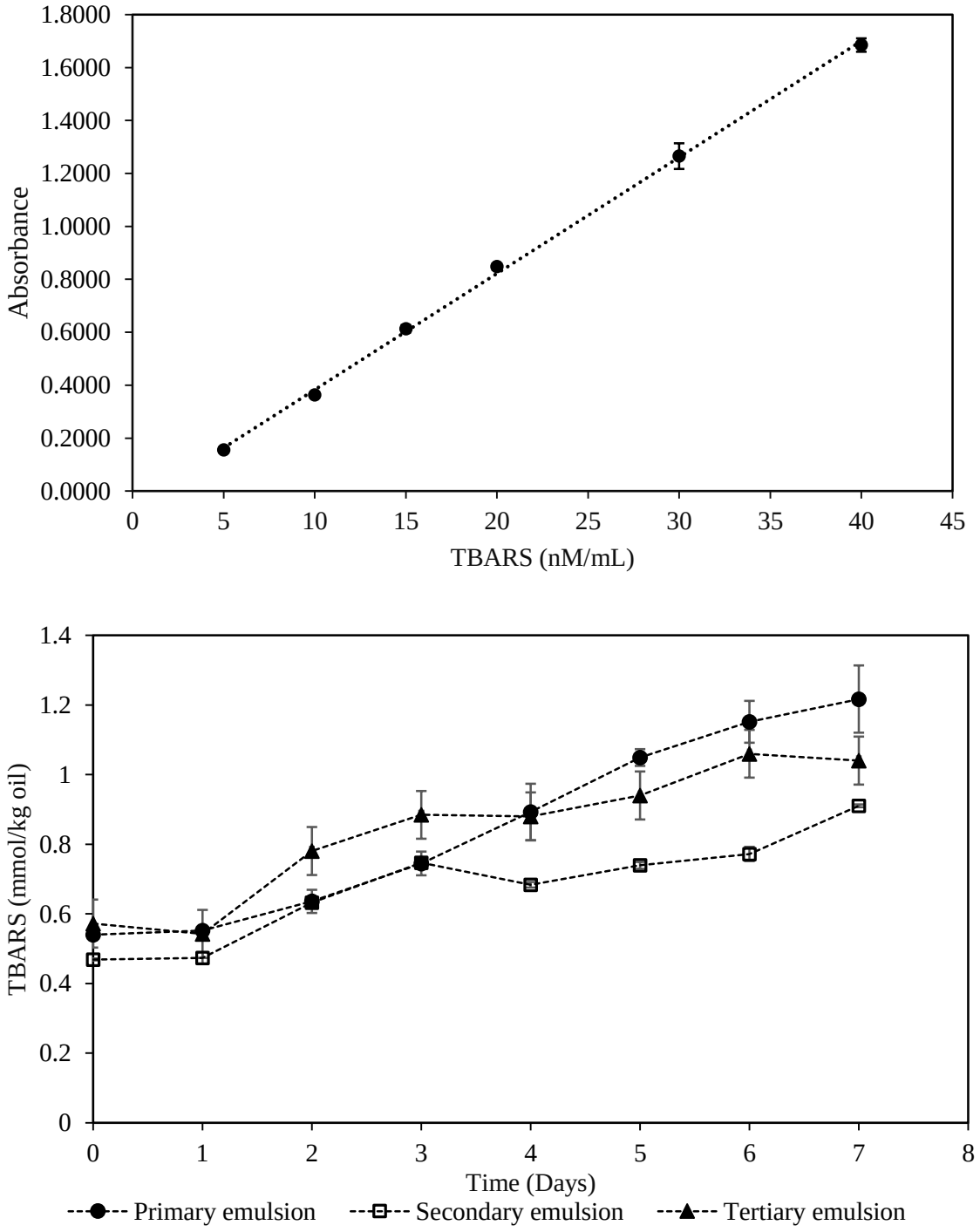


Figure 4.11: (a) Calibration curve used for the determination of concentration of TBARS. (b) Comparison of TBARS concentration detected in primary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate), secondary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate) and tertiary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate + 0.25% w/v chitosan) emulsions at 30 °C. Data represents mean ± standard deviation (n=3).

Primary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate), secondary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate) and tertiary (1% w/v flaxseed oil, 0.4% w/v sodium caseinate + 0.25% w/v sodium alginate + 0.25% w/v chitosan) emulsions were incubated at 30 °C for 7 days with the goal to determine differences in oxidative stability among the samples. The rate of lipid oxidation in primary, secondary and tertiary emulsions were studied simultaneously by detecting the concentration of lipid oxidation markers (hydroperoxides and TBARS) as indicated in figures 4.10 and 4.11.

The rate of lipid hydroperoxide formation was least in secondary emulsion, followed by tertiary emulsion and primary emulsion (Figure 4.10 (b)). The rate of TBARS formation was highest for primary emulsion with a rising trend (Figure 4.11 (b)). On the other hand, secondary and tertiary emulsions exhibited a more or less constant TBARS value after 3 days with tertiary layer producing higher amounts of lipid oxidation products than secondary emulsions. This observation showed that secondary emulsions though negatively charged had a better oxidative stability than cationic primary and tertiary emulsion droplets probably due to thicker and more uniform interfacial membrane. Sodium alginate, being a linear polymer could have contributed to the formation of dense interfacial layer, thus protecting the enclosed oil from oxidation. Generally, it is expected that negatively charged interfacial membranes attracted more pro-oxidants thus enhancing oxidation. However, our observation showed that droplet interface homogeneity and uniform coverage had a greater role in preventing oxidative degradation as anionic secondary emulsions emerged to better protect the enclosed flaxseed oil. The sodium caseinate-sodium alginate barrier prevented the interactions between metal and lipid better. This observation is supported by Hu et al. (2003) and Chaprenet et al. (2014) as they argued that more than positive ζ-potential of droplets, several underlying factors contributed to the interaction between oil phase and aqueous phase components. In addition, structurally homogeneous interfacial films were associated with reduced lipid oxidation (Berton-Carabin et al., 2013). Similar observations were made by Katsuda et al. (2008) where higher oxidative stability of anionic multilayer has been demonstrated in emulsion bilayers of β-lactoglobulin-citrus pectin than cationic monolayer of β-lactoglobulin.

Tertiary emulsion interface having both the favorable attributes of increased interfacial thickness and positive charge was only above primary and but below secondary

emulsions while it was expected to be the finest among primary, secondary and tertiary emulsions in protecting the lipid phase. It is probable that the presence of chitosan in tertiary emulsion interacted with second layer alginate such that the regularity of the interface was perturbed. This made the oil droplets susceptible to oxidation. Results of Gudipati et al. (2010) have shown similarity in lipid oxidation fashion observed in our study in that secondary emulsions had better oxidative stability than primary and tertiary emulsions. Nonetheless, their primary, secondary and tertiary layers were anionic, cationic and anionic respectively. It implied that charge distribution of biopolymers were exactly opposite to that of present study.

Chapter 5

Conclusion and future scope

This research work has shown that it is possible to prepare multilayer emulsions from natural food ingredients such as sodium caseinate, sodium alginate and chitosan. This was achieved by careful selection of biopolymers based on their electrical characteristics (ζ-potential at a given pH and solubility) as well as control of the emulsion preparation procedure (for instance, mixing pH, polyelectrolyte concentration and droplet concentration). Tertiary oil-in-water emulsions containing cationic droplets stabilized by sodium caseinate-sodium alginate-chitosan were designed using layer-by-layer electrostatic deposition of biopolymer over a charged interface. Under certain circumstances, the stability of emulsions could be tremendously enhanced by coating the oil droplets with two or more biopolymer layers. Primary sodium caseinate coated droplets coagulated extensively when salt of 70 mM was added. Sodium caseinate- sodium alginate coated droplets in the secondary emulsion resisted droplet aggregation when held at temperatures ranging from 30 to 90 °C and salt concentration ≤ 70 mM NaCl. Sodium caseinate- sodium alginate-chitosan coated flaxseed oil droplets in the tertiary emulsion resisted droplet aggregation at salt concentration ≤ 70 mM NaCl but emulsion structure collapsed when holding temperature was raised above 40 °C. The improved stability of multilayer emulsions to both varying temperature and salt concentration was attributed to their thick interfacial membranes that did not rupture due to stress factors or to the colloidal interactions among oil droplets such as electrostatic and steric repulsion.

The remarkable oxidative stability of multilayer emulsion droplets in comparison with primary oil globules could be associated with the thick and dense interfacial membranes formed using layer-by-layer electrostatic deposition technique. In general, multilayer emulsion system have excellent potential in delivering bioactive oils such as flaxseed oil that comprise of highly sensitive ω-3 fatty acids into functional foods.

Multilayer emulsions could have several potential applications in various fields of food industry. For instance, the thick interfacial membrane could be used to encapsulate, protect and deliver lipophilic materials such as oil-soluble vitamins and antioxidants or to release flavor in a controlled fashion. On the other hand, these complex emulsion systems could basically be utilized for enhancing the structural stability of emulsions against

stresses (pH, salt and temperature). In circumstances where temperature variations can affect the integrity of protein-stabilized colloidal systems, secondary emulsions can be employed as an alternative. In addition, multilayer emulsions could be tailored to selectively release an active compound in a specific location inside human digestive tract, for instance, the bioactive could be secured in acidic environments of stomach but delivered subsequently in the small intestine. The argument that multilayer emulsions form stable systems at lower pH advocates their suitability to foods including salad dressings, yoghurt drinks and beverages that are predominantly acidic.

The major practical hindrance that obstructs the widespread application of multilayer emulsion technology in the food industry is the prevalence of bridging flocculation. It occurs because droplet-droplet collisions occur faster than the adsorption of biopolymer layers. In order to prevent this destabilization mechanism, limited concentration of lipid has to be used in the formulation of multilayered emulsion so that biopolymer adsorption occurs more rapidly than oil droplet- droplet collision. Furthermore, suitable combination of biopolymers required to obtain the desired interfacial properties in the intended food environment (temperature and salt sensitivity) is of considerable significance.

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Dissemination

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I. Sarkar, P., Irshaan, S., Sivapratha, S., & Choudhary, R. (2016**). Nanotechnology in Food Processing and Packaging.** In *Nanoscience in Food and Agriculture 1* (pp. 185-227). Springer International Publishing.

II. Sivapratha, S., & Sarkar, P. (2016). **Multiple layers and conjugate materials for food emulsion stabilization.** *Critical Reviews in Food Science and Nutrition*, (just-accepted), (Taylor and Francis, Impact factor: 5.492).

III. Sivapratha, S., and Sarkar, P. (2017). **Oxidative stability and effect of stress factors on flaxseed oil-in-water emulsions stabilized by sodium caseinate-sodium alginate-chitosan interfacial membrane.** *Chemical papers* – manuscript under review

Conference:

I. Sivapratha, S., & Sarkar, P. **Comparison of Emulsions Stabilized by Anionic and Cationic Biopolymers**, 3rd *Asian Food Safety and Security Association (AFSA)* conference on 'Food Safety and Food Security', 15th -17th September, 2016.